Lipid biochemistry and physiology of Antarctic krill (*Euphausia superba*) in the present day and under future ocean acidification scenarios

by

Jessica Ericson, BSc, MSc

Institute for Marine and Antarctic Studies

Submitted in fulfilment of the requirements for the Degree of
Doctor of Philosophy in Quantitative Marine Science
University of Tasmania
July 2019

Declaration of originality

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

30th July 2019

Jessica Ericson Date

Statement of authority of access

The publishers of the papers comprising Chapters 2 and 4 hold the copyright for that content. Access to the material should be sought from the respective journals. Any non-published content of the thesis may be made available for loan and limited copying in accordance with the Copyright Act 1968.

30th July 2019

Jessica Ericson Date

Statement of co-author contributions

The following collaborators and institutions contributed to the publication of the work undertaken as part of this thesis:

Candidate: Jessica Ericson, Institute for Marine & Antarctic Studies
Author 1: Nicole Hellessey, Institute for Marine & Antarctic Studies
Author 2: Peter Nichols, CSIRO Oceans and Atmosphere
Author 3: So Kawaguchi, Australian Antarctic Division
Author 4: Stephen Nicol, Institute for Marine & Antarctic Studies
Author 5: Nils Hoem, Aker Biomarine
Author 6: Patti Virtue, Institute for Marine & Antarctic Studies

Chapter 2

Paper 1: Ericson JA, Hellessey N, Nichols PD, Kawaguchi S, Nicol S, Hoem N, Virtue P (2018). Seasonal and interannual variations in the fatty acid composition of adult *Euphausia superba* Dana, 1850 (Euphausiacea) samples derived from the Scotia Sea krill fishery. *Journal of Crustacean Biology*, **38** (6): 656 – 661. doi: 10.1093/jcbiol/ruy032.

Author Contributions:

Candidate was the primary author and was responsible for the research idea, formalisation, development, laboratory and data analyses and manuscript preparation. Author 1 assisted with selected laboratory analyses. Authors 2 and 6 contributed to project design and development, and editing of the manuscript. Authors 1, 3, 4 and 5 provided research guidance and assisted with editing of the manuscript.

Chapter 3

Paper 2: Ericson JA, Hellessey N, Nichols PD, Nicol S, Kawaguchi S, Hoem N, Virtue P (under review for *Polar Biology*). New insights into the seasonal diet of Antarctic krill using triacylglycerol and phospholipid fatty acids.

Author Contributions:

Candidate was the primary author and was responsible for the research idea, formalisation, development, laboratory and data analyses and manuscript preparation. Author 1 assisted with selected laboratory analyses. Authors 2 and 6 contributed to project design and development, and editing of the manuscript. Authors 1, 3, 4 and 5 provided research guidance and assisted with editing of the manuscript.

Chapter 4

Paper 3: Ericson JA, Hellessey N, Kawaguchi S, Nicol S, Nichols PD, Hoem N, Virtue P (2018). Adult Antarctic krill proves resilient in a simulated high CO_2 ocean. *Communications Biology*, **1**: 190. doi: 10.1038/s42003-018-0195-3.

Author Contributions:

Candidate was the primary author and was responsible for the research idea, formalisation, development, laboratory and data analyses and manuscript preparation. Author 1 assisted with selected laboratory analyses. Authors 3 and 6 contributed to project design and development, and editing of the manuscript. Authors 1, 2, 4 and 5 provided research guidance and assisted with editing of the manuscript.

Chapter 5

Paper 4: Ericson JA, Hellessey N, Kawaguchi S, Nichols PD, Nicol S, Hoem N, Virtue P (under review for *Scientific Reports*). Near-future ocean acidification does not alter the lipid content and fatty acid composition of adult Antarctic krill.

Author Contributions:

Candidate was the primary author and was responsible for the research idea, formalisation, development, laboratory and data analyses and manuscript preparation. Author 1 assisted with selected laboratory analyses. Authors 2, 3 and 6 contributed to project design and development, and editing of the manuscript. Authors 1, 4 and 5 provided research guidance and assisted with editing of the manuscript.

We the undersigned agree with the above stated proportion of work undertaken for each of the above published (or submitted) peer-reviewed manuscripts contributing to this thesis:

……..

Associate Professor Patti Virtue
Primary Supervisor
Institute for Marine and Antarctic Studies
University of Tasmania
Date: 30th July 2019

……..

Professor Craig Johnson
Head of Centre, Ecology & Biodiversity
Institute for Marine and Antarctic Studies
University of Tasmania
Date: 30th July 2019

List of publications & awards obtained during candidature

Publications

Ericson JA, Hellessey N, Nichols PD, Kawaguchi S, Nicol S, Hoem N, Virtue P (2018a). Seasonal and interannual variations in the fatty acid composition of adult *Euphausia superba* Dana, 1850 (Euphausiacea) samples derived from the Scotia Sea krill fishery. *Journal of Crustacean Biology* **38** (6): 656 – 661.

Ericson JA, Hellessey N, Kawaguchi S, Nicol S, Nichols P, Hoem N, Virtue P (2018b). Adult Antarctic krill proves resilient in a simulated high CO_2 ocean. *Communications Biology* **1:** 190. doi: 10.1038/s42003-018-0195-3.

Hellessey N, **Ericson** JA, Nichols PD, Kawaguchi S, Nicol S, Hoem N, Virtue P (2018). Seasonal and interannual variation in the lipid content and composition of *Euphausia superba* Dana 1850 (Euphausiacea) samples derived from the Scotia Sea fishery. *Journal of Crustacean Biology* **38** (6): 673 – 681.

Hellessey N, **Ericson** JA, Nichols PD, Kawaguchi S, Nicol S, Hoem N, Virtue P (under review for *Limnology and Oceanography*). Regional variability of Antarctic krill (*Euphausia superba*) diet as determined using lipid, fatty acid and sterol composition.

Awards

2019 Ericson *et al.* 2018b chosen as an "editors pick" for the Communications Biology 1st Year Anniversary Collection.

2018 Institute for Marine and Antarctic Studies Best Student Publication Award (for Ericson *et al.* 2018b).

Marine Ecosystem Assessment for the Southern Ocean Conference Oral Presentation – Highly Commended Award.

2017 Student Bursary for Conference Travel to attend the Third International Symposium on Krill ($500 AUD).

Institute for Marine and Antarctic Studies Conference and Research Travel Scholarship to attend the Third International Symposium on Krill in St Andrews, Scotland ($2263 AUD).

Australian Marine Sciences Association (AMSA) Allen Award – Highly Commended.

2016 Antarctic Climate & Ecosystems Cooperative Research Centre Conference – Second Prize for Poster Presentation ($500 AUD).

2015 University of Tasmania Australian Postgraduate Award ($26,682 AUD per annum).

CSIRO-UTAS Quantitative Marine Science Top-Up Scholarship ($5000 AUD per annum).

Acknowledgements

First and foremost I would like to thank Patti Virtue, Peter Nichols, So Kawaguchi, Steve Nicol and Nils Hoem - my amazing team of supervisors. I feel so lucky and fortunate to have had such an encouraging and knowledgeable group who really went over and above in their supervisory roles. Thank-you for the unwavering support and assistance that you have given me during my candidature, which I will never take for granted. To my krill buddy Nicole Hellessey – thanks for sharing all the ups and downs that we've had navigating the PhD path side by side. We made a great team, and I look forward to following all your successes into the future. To Tasha, Ash, Blair and Rob at the Antarctic Division Krill Aquarium – thanks for letting me into your space for a whole year, and being there to assist whenever I needed you. To Andy Revill, Peter Mansour and Mina Brock, I really appreciate all the time you took out of your busy days to answer my queries during my laboratory work at CSIRO, especially since it was almost always at short notice! Thank you to the employees of Aker Biomarine and the crew of the FV Saga Sea; some of you I have met and some I have not. It was a pleasure to work with an industry that is so interested in, and supportive of, science. I also acknowledge the ARC Linkage Grant and my Australian Postgraduate Award, which made this research possible.

Thank-you to the many other IMAS staff and students, particularly Lavy Ratanarajah and my BOTES executive team for all the fun times in between thesis writing. To the Hobart mountain bike community and my ocean swimming friends – thanks for being my honorary family whilst in Tassie, and for all the outdoor adventures we've had together in this beautiful State. Thank you to my NZ friends who have been there whenever I needed to chat, and to those that have visited and given me little breaks from PhD study.

To Mum, Dad and Sam – once again, thank you for your never-ending support and encouragement throughout my PhD and during my life in general. I am so lucky to have a family who whole heartedly believes in me and supports me unconditionally. Nik – thank you for taking a huge leap of faith and moving over to Hobart with me to support me on my PhD journey. Your love, positivity, sense of humour and easy-going nature are just what I have needed.

Lastly, thanks to the Antarctic krill for being such an interesting and fascinating organism to study. I hope this research does you justice.

Dedication

I dedicate this thesis to my late grandfather Ron Power. You once said to me that ‘mankind is rather deluded in believing that he represents the peak of life’s complexity’. I know you would’ve read this thesis from front to back and marvelled over the amazing complexity of the Antarctic krill. I feel honoured to stand on your shoulders and ensure that the biologist gene in the family does carry on to the F2 generation, like you hoped. You deserve an honorary doctorate for all the scientific knowledge that you acquired over your colourful life.

So this one’s for us, Grandad.

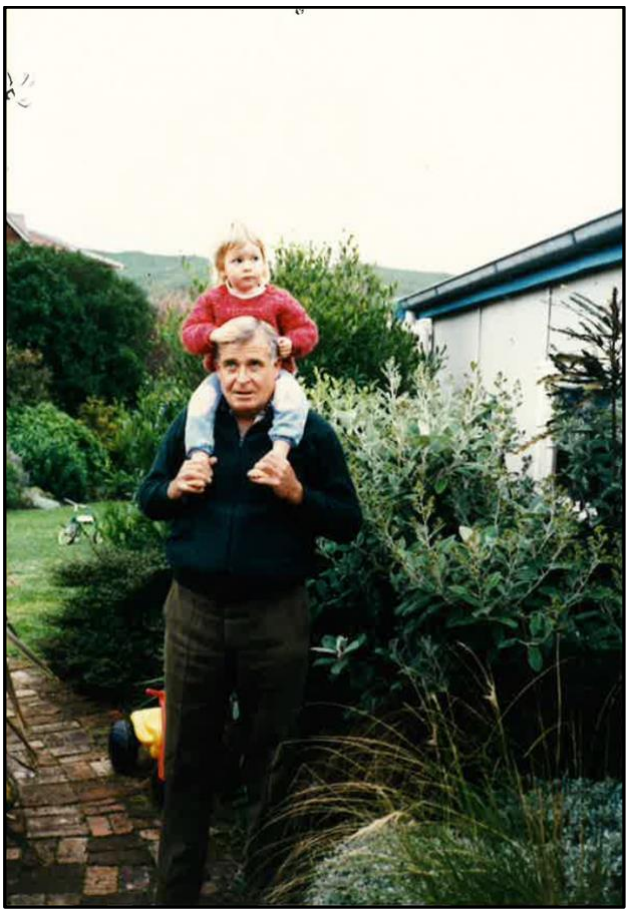


Table of Contents

List of Figures

Figure 2.3. Principal component analysis of fatty acid percentage composition of *Euphausia superba* in 2014 (**A**), 2015 (**B**), and 2016 (**C**).

Figure 2.4. Ratios of 20:5n-3/22:6n-3 and 16:1n-7c/18:4n-3 (diatom/flagellate ratios), 18:1n-9c/18:1n-7c (carnivory/herbivory ratio), and Σ 20:1 + 22:1 isomers (copepod dietary indicator) in *Euphausia superba* in different locations and years (mean ± SE). WAP, West Antarctic Peninsula, SOI, South Orkney Islands, SG, South Georgia. The sample size (*n*) for each location and year combination is provided. The seasons in which krill were sampled in each location and year are also provided (Su, summer; A, autumn; W, winter; Sp, spring). Significant differences ($p < 0.05$) between locations and years are denoted by points that do not share lower case letters.

Figure 2.1. Map of locations where krill samples were collected by the krill fishery in (○) summer (Jan – Feb), (△) autumn (Mar – May), and (+) winter/spring (Jun – Sep) of 2016, at the West Antarctic Peninsula (WAP), South Orkney Islands (SOI) & South Georgia (SG). Map modified from Hellessey *et al.* (2018).

Figure 3.2. Principal components analysis (PCA) of the fatty acid composition (%) of triacylglycerols (TAG) & phospholipids (PL) of male *Euphausia superba*. Only fatty acids >0.5% were used for PCA. Data for krill from all seasons are combined.

Figure 3.3. Percentages (mean ± SE) of fatty acid biomarkers 20:5n-3, 22:6n-3, 18:4n-3, 16:1n-7c, ∑ 20:1 + 22:1, & ratios of 18:1n-9c/18:1n-7c in male *Euphausia superba*, in triacylglycerols (TAG) & phospholipids (PL). Data from all seasons are combined. Significant differences between the TAG and PL are denoted by asterisks where * = $p < 0.05$, ** = $p < 0.001$. For each lipid class $n = 3$.

Figure 3.4. Percentages (mean ± SE) of fatty acid biomarkers 20:5n-3, 22:6n-3, 18:4n-3, 16:1n-7c, ∑ 20:1 + 22:1, & ratios of 18:1n-9c/18:1n-7c in triacylglycerols (TAG) & phospholipids (PL) of male *Euphausia superba*, in summer (Jan – Feb), autumn (Mar – May) & winter/spring (Jun – Sep) of 2016. For each lipid class & season $n = 12$.

Figure 4.1. Survival (%) of *Euphausia superba* in each experimental week in 400 (present day control), 1000, 1500, 2000 and 4000 µatm pCO_2 seawater.

Figure 4.2. Total length (mm) of *Euphausia superba* in 400, 1000, 1500, 2000 and 4000 µatm pCO_2 seawater in weeks 1, 2, 4, 5, 26, 39, 41, 43 and 46 of the experiment. Box plot elements are centre line, median; box limits, upper and lower quartiles; whiskers extend to the most extreme data point no more than 1.5 times the interquartile range. Shaded black dots denote outlier values that are over 1.5 times the interquartile range. Months and seasons corresponding to the experimental weeks are also provided (X-axis). For each pCO_2 by week combination $n = 2 – 10$. See Appendix VIIA for exact sample sizes for each treatment combination.

Figure 4.3. Triacylglycerol (mg g^{-1} dry mass; DM) of *Euphausia superba* in 400, 1000, 1500, 2000 and 4000 µatm pCO_2 seawater in weeks 1, 2, 4, 5, 26, 39, 41 and 43 of the experiment. Box plot elements are centre line, median; box limits, upper and lower quartiles; whiskers extend to the most extreme data point no more than 1.5 times the interquartile range. Shaded

black dots denote outlier values that are over 1.5 times the interquartile range. Months and seasons corresponding to the experimental weeks are also provided (X-axis). For each pCO_2 by week combination $n = 3 – 5$. See Appendix VIIB for exact sample sizes for each treatment combination.

Figure 4.4. Maturity scores (**A**) and ovary stages (**B**) of *Euphausia superba* in 400, 1000, 1500, 2000 and 4000 µatm pCO_2 seawater. Maturity scores are the average scores (mean ± SE) of krill in each treatment for weeks 39, 41, 43 and 46 combined ($n = 5$ for the 400 – 2000 µatm pCO_2 treatments and $n = 3$ for the 4000 µatm pCO_2 treatment). Ovary stages are the average ovary stages (mean ± SE) of female krill in week 46 only. The physical ovary stages relating to each stage number are also provided (2 = oogenesis, 3 = previtellogenesis (cell size < 200 µm), 4 = previtellogenesis (cell size > 200 µm), 5 = early vitellogenesis). Higher maturity scores and ovary stages indicate a more advanced reproductive stage. For 400 – 2000 µatm pCO_2 treatments $n = 8$, and for the 4000 µatm pCO_2 treatment $n = 2$.

Figure 4.5. Respiration rates (µl O_2 mg dry mass hr $^{-1}$) of *Euphausia superba* in 400, 1000, 1500, 2000 and 4000 µatm pCO_2 seawater in experimental week 38 (early spring). Each black point denotes the respiration rate of an individual krill ($n = 10$ for each pCO_2 treatment). Blue circles denote the mean for each pCO_2 treatment, and error bars represent the 95% confidence intervals for the mean.

Figure 4.6. Haemolymph pH of *Euphausia superba* in 400, 1000, 1500, 2000 and 4000 µatm pCO_2 seawater in experimental week 46. Each black point denotes the haemolymph pH of an individual krill ($n = 5$ for each pCO_2 treatment). Blue circles denote the mean for each pCO_2 treatment, and error bars represent the 95% confidence intervals for the mean. Seawater pH values are shown below pCO_2 treatments for ease of interpretation.

Figure 5.1. (A) Total lipid (mg g $^{-1}$ dry mass; mean ± SE) and **(B)** phospholipid/sterol ratio (mean ± SE) of *Euphausia superba* in weeks 1, 2, 4, 5, 26, 39, 41 and 43 of the one-year ocean acidification experiment. For each pCO_2 treatment and week $n = 3 – 7$. Statistically significant differences to ambient seawater (400 µatm pCO_2) are shown with an asterisk ($p < 0.05$).

Figure 5.2. Principal component analyses of the fatty acid percentage composition of Euphausia superba in 400, 1000, 1500, 2000 and 4000 µatm pCO_2 during **(A)** weeks 1 – 5 (summer) and **(B)** weeks 26 – 43 (winter and spring) of the one-year ocean acidification experiment. The amount of variation (%) explained by each principal component (PC) is shown on the x-axis (PC1) and y-axis (PC2).

Figure 5.3. Fatty acid indicators of homeoviscous adaptation and immune responses in *Euphausia superba* exposed to 400, 1000, 1500, 2000 and 4000 µatm pCO_2 in experimental weeks 26, 39, 41 and 43, where **(A)** Mean chain length (MCL); (**B**) ratio of polyunsaturated to saturated fatty acids (PUFA/SFA); (**C**) ratios of docosahexaenoic acid/arachidonic acid (22:6n-3/20:4n-6); (**D**) alpha-linolenic acid/linoleic acid (18:3n-3/18:2n-6). All values are mean ± SE.

List of Tables

Abstract

Antarctic krill (*Euphausia superba*, hereafter 'krill') are lipid-rich euphausiids with an important role in the Southern Ocean, including as the primary prey of Antarctic megafauna (whales, seals, penguins), fish, squid and seabirds. They contain high levels of nutritious long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFA), specifically eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3). The sheer abundance of krill in the Southern Ocean means that the ecosystem is largely driven by energy derived from krill lipids. In addition to their ecological importance, a Scotia Sea krill fishery harvests krill, including for commercial use of their LC-PUFA. The existence of this year-round krill fishery provides a unique opportunity to collect krill samples for research over large spatial and temporal scales, which is unfeasible using scientific research vessels.

In this thesis, fishery caught krill samples were used to investigate the fatty acid content and composition of krill, during all seasons and over consecutive years (2013 – 2016). This research (presented in Chapter 2) aimed to fill knowledge gaps on the seasonal diet of krill (particularly in winter) in the Scotia Sea region, using fatty acids as dietary biomarkers. Krill were primarily herbivorous in summer (higher levels of 20:5n-3 and 22:6n-3, and low 18:1n-9c/18:1n-7c ratios) and became more omnivorous from autumn to spring (increasing ratios of 18:1n-9c/18:1n-7c and percentages of Σ 20:1 + 22:1 isomers). Seasonal proportions of herbivory and omnivory differed between years, and fatty acid composition differed between fishing locations. Selected samples were also used to investigate the composition of fatty acids in the structural (phospholipids) and storage lipids (triacylglycerols) of krill (Chapter 3). Triacylglycerol fatty acids (thought to better represent recent diet), reflected omnivorous feeding with highest percentages of flagellate biomarkers (18:4n-3) occurring in summer, diatom biomarkers (16:1n-7c) from autumn-spring, and greater carnivory (higher ∑ 20:1 + 22:1 and 18:1n-9c/18:1n-7c ratios) in autumn. Phospholipid fatty acids were less variable and were higher in the essential membrane fatty acids 20:5n-3 and 22:6n-3. Percentages of the major krill sterol, cholesterol, were significantly higher in winter and spring compared with summer and autumn. Results presented in Chapters 2 and 3 highlighted the dynamic nature of krill lipids, and the flexible diet of krill, which likely contributes to their huge biomass and success as one of the most abundant organisms on Earth.

Because krill are so important in the Southern Ocean food web, any decreases in krill biomass could result in a major ecological regime shift. Very little is known about how climate change will affect krill. Increasing anthropogenic carbon dioxide (CO_2) emissions are causing ocean acidification, as absorption of atmospheric CO_2 in seawater alters ocean chemistry. Ocean acidification increases mortality and negatively affects physiological functioning in some marine invertebrates, and is predicted to occur most rapidly at high latitudes. Long-term laboratory studies are needed to understand how keystone species such as krill may respond to predicted future pCO_2 levels. A long term experiment was conducted to test whether rising ocean pCO_2 is likely to impact krill physiology and biochemistry (Chapters 4 and 5). Adult krill were exposed to near-future ocean acidification (1000 – 2000 μatm pCO_2) for one year in the laboratory. Krill reared in near-future pCO_2 conditions were able to survive, grow, store fat, mature, and maintain normal respiration rates. Haemolymph pH, lipid and fatty acid composition were also maintained at the same levels as krill in ambient pCO_2 (400 μatm). Negative effects on physiology and lipid biochemistry were only observed in extreme pCO_2 conditions (4000 μatm), which krill will not experience in the wild. These results place adult krill among the most resilient species in ocean acidification studies to date.

In summary, results in this thesis highlight the remarkable adaptability of krill in a changing environment, from short-term seasonal or annual scales, to longer-term decadal scales. Their flexible phenotype may aid their survival in an ocean that is rapidly changing with increasing anthropogenic CO_2 emissions. The data obtained in this thesis can be used for fisheries management to guide fishing activities, and in fisheries models to predict how krill biomass may be affected by climate change. Krill lipid energy fuels the Southern Ocean ecosystem and to date, lipid data has not been included in Antarctic ecosystem models. The large scale of lipid data in this study makes it ideal for inclusion in such models, and it has important implications for the health of the wider Southern Ocean ecosystem.

1 General Introduction

1.1. An introduction to Antarctic krill and their environment

Antarctic krill (*Euphausia superba*, hereafter 'krill') are abundant euphausiids found in the oceans surrounding the Antarctic continent. They grow up to 60 mm in length (Reiss 2016) and have an estimated life span of four to ten years (Pyper 2002; Thomas & Ikeda 1987). Krill are found south of the Polar Front at approximately 55°S (Siegel & Watkins 2016), which marks the boundary between cold fresh Antarctic seawater and warm salty sub Antarctic waters (Freeman *et al.* 2016). They are found in seawater temperatures between -1.8°C and +5°C, but prefer oxygenated coastal seawater between -1.5°C and +1.5°C, that is high in chlorophyll and nutrients (Cuzin-Roudy *et al.* 2014). Krill aggregate in swarms up to 2 million tons in weight and 100km^2 in size (Tarling & Fielding 2016) and migrate throughout the water column, from the surface to depths of at least 3500 m (Clarke & Tyler 2008). Higher densities of krill are often located in the upper 200 m of the water column and in areas with steep bathymetry (Siegel and Watkins 2016). This swarming behaviour and patchy distribution make their biomass difficult to quantify (Atkinson *et al.* 2012), but current estimates of krill biomass range between 60 – 420 million metric tons (Nicol *et al.* 2000; Atkinson *et al.* 2009).

Krill are a keystone species in the Antarctic food web (Figure 1.1), as highly nutritious prey for seabirds, penguins, fish, squid, seals and whales (Trathan & Hill 2016). They feed on a range of phytoplankton and zooplankton, and transfer energy through the food chain when they are consumed by higher predators (Murphy *et al.* 2007). This importance of krill in the Southern Ocean ecosystem has led to krill being the major focus of scientific studies, beginning with the 'Discovery' expeditions in the 1920s (Marr 1962; Siegel 2016b). In the 57 years since Marr published his detailed account of the distribution, behaviour, diet and reproduction of krill (Marr 1962), scientists have conducted voyages to the Southern Ocean to collect krill

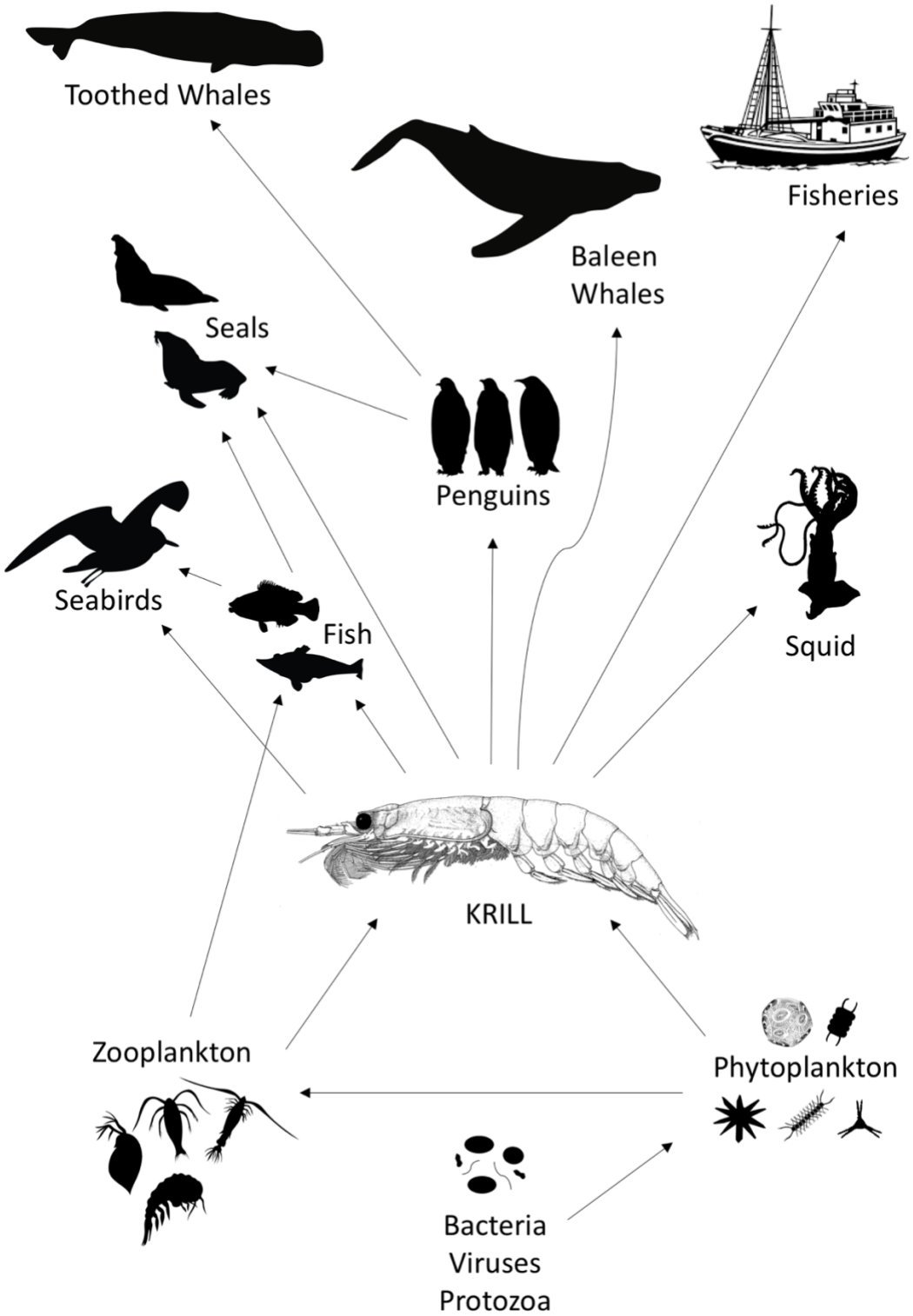


Figure 1.1. A simplified Southern Ocean food web showing the trophic relationships between different taxa, and the importance of krill (*Euphausia superba*) as a keystone species. Images not to scale. Image of *Euphausia superba* taken from Baker *et al.* (1990).

samples and to study the complex life history of this species. Large scale field studies have provided further information on the abundance of krill (Atkinson *et al.* 2017), their length-frequency distributions (Reiss 2016) and the variable sex ratios of swarms (Watkins *et al.* 1992). Modern laboratories and aquarium developments have allowed scientists to study the biology krill for extended periods. A range of techniques have been recently used to study the krill genome (Sales *et al.* 2017), measure the age of krill using their eye stalks (Kilada *et al.* 2017), examine how krill process microplastics (Dawson *et al.* 2018) and observe the behaviour and reproduction of krill in the laboratory (Kawaguchi *et al.* 2010; Kawaguchi 2016).

The majority of scientific voyages to study krill or collect krill samples have been conducted in summer, due to a range of logistical constraints that restrict year-round operation of scientific research vessels (Kawaguchi & Nicol 2007). As a consequence, most of the data collected on krill biology is from the summer months. We have learnt from the limited seasonal studies that are available, that krill have a wide range of physiological adaptations to survive the extreme seasonality of the Southern Ocean. The metabolism, growth, reproduction and diet of krill are all primarily controlled by seasonal cycles and photoperiod (seasonal light regime) (Höring *et al.* 2018). The importance of seasonal cycles in the life history of krill requires further investigation, as the seasonal data collected to date is sparse. The habitat of krill will also be altered by anthropogenic climate change, and we currently have very limited knowledge of how krill may respond to a changing ocean (Flores *et al.* 2012; Meyer & Teschke 2016). Studies that investigate the seasonal and interannual changes in the life cycle of krill, and their response to anthropogenic climate change, are needed to understand the present ecological status and future fate of krill in the Southern Ocean.

1.2. Lipid biochemistry and fatty acids as dietary biomarkers

Lipids are the biochemical building blocks of fats and oils, and comprise a range of compounds that are insoluble in water (Nelson & Cox 2012). They have a variety of roles in organisms; they can be mobilised for use during reproductive processes, used to regulate buoyancy, and are broken down into smaller subunits to provide energy (Kattner *et al.* 2007).

In most organisms, lipids are generally made up of fatty acids (Figure 1.2), which are carboxyl groups with chains of carbon and hydrogen atoms that can be saturated (SFA; contain only single bonds) or unsaturated (contain at least one double bond) (Nelson & Cox 2012). The unsaturated fatty acids can be further divided into two groups; monounsaturated fatty acids (MUFA) which contain one double bond, and the polyunsaturated fatty acids (PUFA) which contain more than one double bond (Figure 1.2). Omega 3 PUFA are essential for organism function as they maintain cell membrane fluidity, and are vital for an animal's survival, energy storage, reproduction and growth (Parrish 2013).

Lipids can be further separated into different classes which include neutral lipids (triacylglycerols and wax esters), polar lipids (phospholipids), sterols, free fatty acids, hydrocarbons and diacylglycerols. Some examples of major lipid classes are shown in Figure 1.3. Phospholipids are comprised of two fatty acids and a phosphate group attached to a glycerol backbone, while triacylglycerols are comprised of three fatty acids connected to a glycerol backbone (Figure 1.3). Sterols are characterized by a four-ring system and a side chain that determines the sterol type (Figure 1.3).

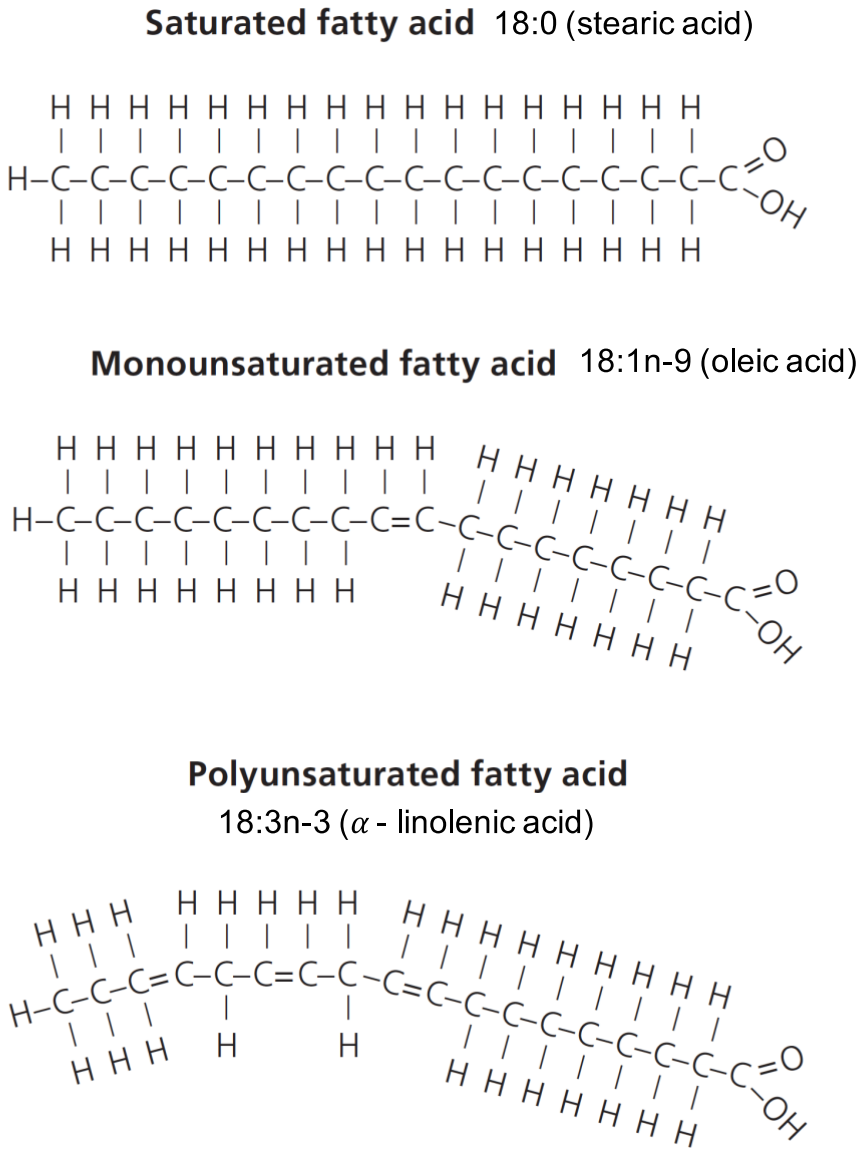


Figure 1.2. Some examples of the molecular structures of saturated fatty acids (SFA), mono-unsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). Figure modified from Figure 1 in White (2009).

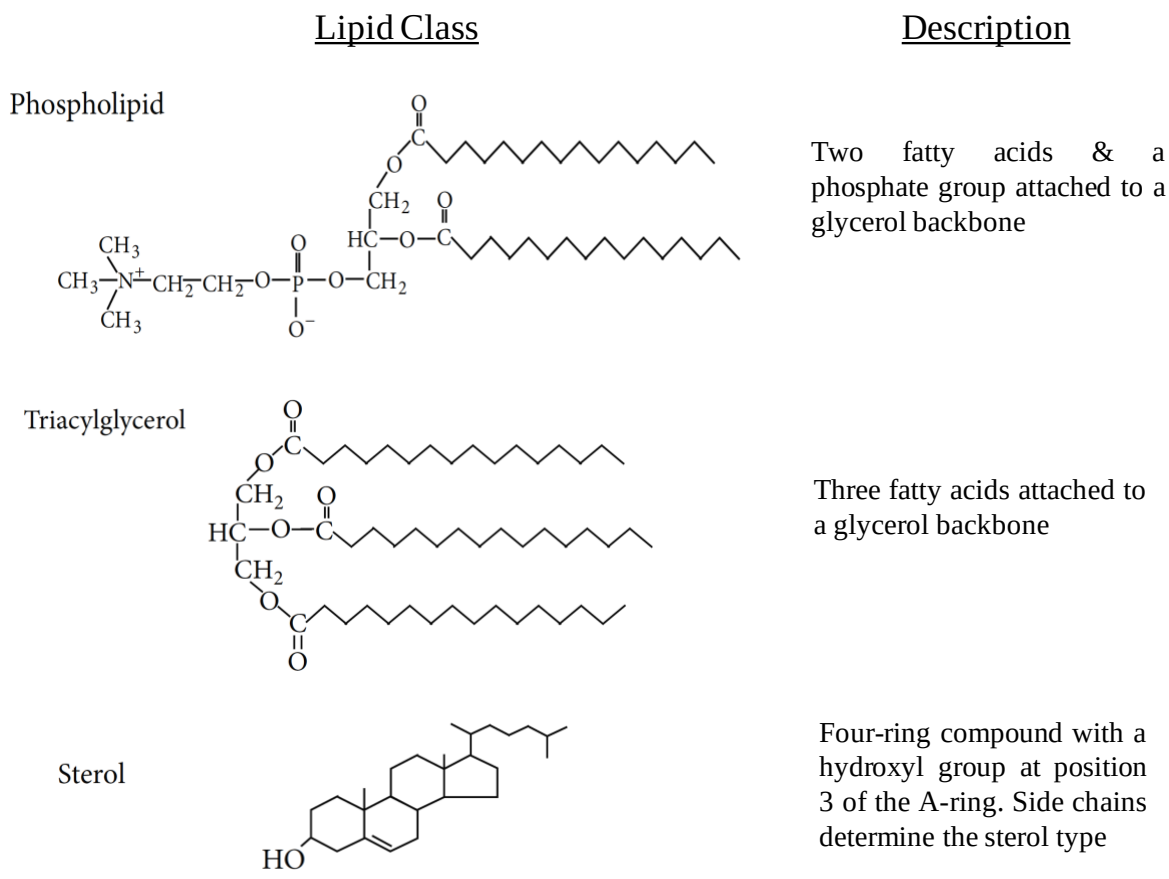


Figure 1.3. Biochemical structure of a selection of important lipid classes, illustrating an example of a phospholipid, triacylglycerol and a sterol. Diagram modified from Figure 1 in Parrish (2013).

Triacylglycerols and wax esters function as the primary energy store in organisms, while phospholipids are a vital component in the structure of cell membranes (Dalsgaard *et al.* 2003; Kattner *et al.* 2007; Nelson & Cox 2012). Some phospholipids (such as phosphatidylcholine in krill) can also be used for energy storage (Kattner *et al.* 2007). Sterols are vital components of cell membranes, important precursors for hormone synthesis, and are necessary for growth and survival (Kanazawa 2001; Martin-Creuzburg & von Elert 2009)

Marine zooplankton accumulate lipid stores from the prey that they consume, and zooplankton in polar regions have particularly large quantities of lipids (Kattner & Hagen 1995; Kattner *et al.* 2007). The percentages and quantities of lipids in polar zooplankton vary

seasonally, reflecting seasonal fluctuations in concentrations of their prey, and physiological demands for energy (Hagen & Schnack-Schiel 1996; Hagen *et al.* 1996). Lipids in Antarctic copepods and krill can exceed 40% of their dry weight (Hagen & Schnack-Schiel 1996; Hagen *et al.* 2001), which make them a high quality energy-dense prey for higher predators, particularly during summer and autumn.

Some fatty acids can only be obtained from the diet, as organisms either have a limited or no ability to synthesize them de-novo (Dalsgaard *et al.* 2003). Fatty acids that are primarily derived from the diet can be used as biomarkers, to obtain information about an organism's likely prey consumption (Dalsgaard *et al.* 2003; Bergé & Barnathan 2005). These biomarkers can be used alone or in conjunction with other techniques such as stable isotopes and stomach content examination to make inferences about an organism's trophic level and feeding behaviour (Schmidt *et al.* 2006). Sterols can also be used as dietary biomarkers, as minor sterols found in marine invertebrates often derived from specific phytoplankton classes (Parrish *et al.* 2000).

1.3. Anthropogenic impacts on krill

1.3.1. Krill fishery

Antarctic krill are commercially fished for a range of products including their oil containing omega 3 fatty acids, and to produce aquaculture meal. The main market for 'krill oil' nutraceutical products is for human consumption (Nicol *et al.* 2012). Fishing for krill began in 1961 and the fishery grew rapidly in the 1970's and 1980's (Nicol & Endo 1999). The Convention for the Conservation of Antarctic Marine Living Resources (CCAMLR) was enacted in 1980 to regulate the fishery and prevent the over-exploitation of krill (Constable *et al.* 2000). The convention operates using a precautionary approach, by setting a trigger level

(currently 620,000 tons) that cannot be exceeded until further research is conducted into the impacts of fishing (CCAMLR 2017). Fishing in specific areas with high densities of krill predators is also even more tightly regulated (Nicol *et al.* 2012).

The largest historical catch was 528,000 tons in 1982, and under CCAMLR management this declined to an annual average catch of 388,000 tons between 1986 – 1991 (Nicol *et al.* 2012). Norway and Korea are now the major krill fishing nations (taking 41% and 21% of the annual catch respectively) (CCAMLR 2015) and current annual catches are approximately 250,000 tons year $^{-1}$ (CCAMLR 2017). Krill fishing primarily occurs in three CCAMLR statistical subareas: the West Antarctic Peninsula (Subarea 48.1), the South Orkney Islands (Subarea 48.2) and South Georgia (Subarea 48.3) (Figure 1.4). In recent years some exploratory fishing has also been carried out in the Indian Ocean sector (Subareas 58.4.1 and 58.4.2) of the Southern Ocean (CCAMLR 2017).

Concerns have been raised about the effects of fishing on krill predators, and studies have been conducted to assess the impact of the fishery on higher trophic levels (Butterworth & Thomson 1995; Mangel & Switzer 1998; Alonzo *et al.* 2003; Plagányi & Butterworth 2012; Weinstein *et al.* 2017). Current low levels of fishing pressure are unlikely to have a significant impact on the krill population, but the fishery needs to be carefully managed into the future if the fishery expands (Nicol *et al.* 2012).

Fishing vessels can also be used to collect samples for scientific research, and this is particularly valuable in remote locations where sampling is difficult. Scientific research vessels face a range of logistical constraints in Antarctica, so the krill fishery (which operates year-round) has been identified as a potential and valuable source of samples (Kawaguchi & Nicol 2007).

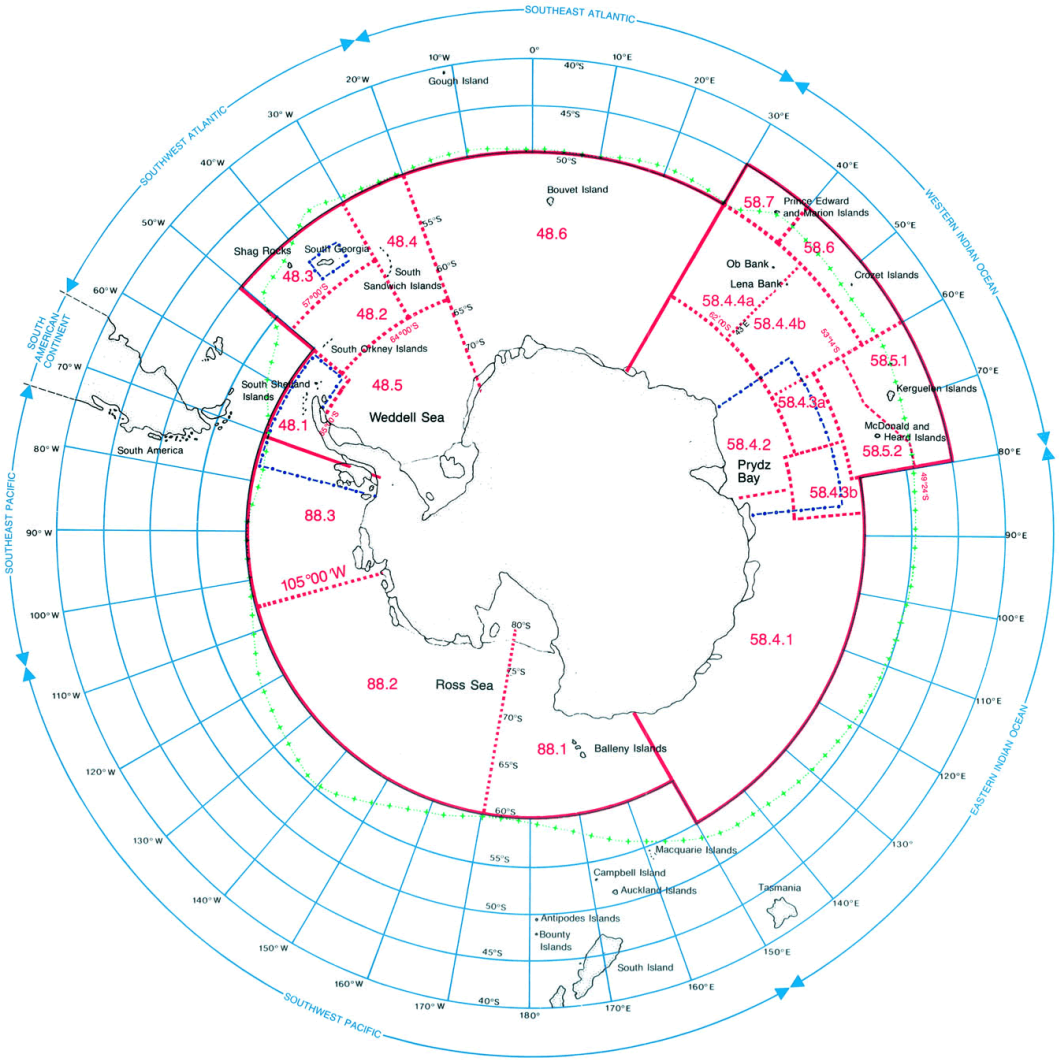


Figure 1.4. Map of the Convention for the Conservation of Antarctic Marine Living Resources (CCAMLR) fishing areas and subareas. Solid red lines denote boundaries of statistical areas, while dashed red lines denote boundaries of statistical subareas. The green line indicates the location of the Antarctic convergence and dark blue lines denote the boundaries of CCAMLR integrated study regions. Map downloaded and modified from http://archive.ccamlr.org/pu/E/conv/map.htm.

1.3.2. Climate change and ocean acidification

Increasing anthropogenic carbon dioxide (CO_2) emissions are altering the Earth's climate, as elevated atmospheric CO_2 levels enhance the 'greenhouse effect' and warm the Earth's surface (IPCC 2014). Anthropogenic climate change is causing an increase in extreme weather events,

ocean warming and sea level rise, which will have consequences for humans and other terrestrial and marine organisms (IPCC 2014). Despite their current relative isolation from the industrial world, organisms in the Southern Ocean will be faced with increased human presence and an array of climate change stressors if anthropogenic CO_2 emissions are not mediated (Rintoul *et al.* 2018) (Figure 1.5). These stressors include increasing seawater temperatures, sea ice loss, biological invasions, changes in ecosystem structure and ocean acidification (Figure 1.5).

Ocean acidification is a major 'side-effect' of increasing anthropogenic CO_2 emissions, often referred to as the 'other' CO_2 problem (Doney *et al.* 2009). Approximately 30% of anthropogenic CO_2 emissions are sequestered in ocean surface waters (Hoegh-Guldberg & Bruno 2010), and as CO_2 is dissolved in seawater it causes a series of chemical reactions (Equation 1) that cause an increase in seawater CO_2 (pCO_2), and a net decrease in seawater pH and carbonate ions (CO_3^{2-}):

$CO_{2\ (atmos)} \leftrightarrow CO_{2\ (aq)} + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-}$ (Doney *et al.* 2009) (Equation 1)

Atmospheric CO_2 levels have increased by over 99 µatm since the industrial revolution (Brewer 2009; Ekwurzel *et al.* 2017), corresponding to a decrease in seawater pH of 0.1 pH units (Hoegh-Guldberg & Bruno 2010). If emissions are not mediated, atmospheric CO_2 is predicted to exceed 1900 µatm by the year 2300, causing a further decrease in seawater pH of 0.77 units (Caldeira & Wickett 2003) (Figure 1.6).

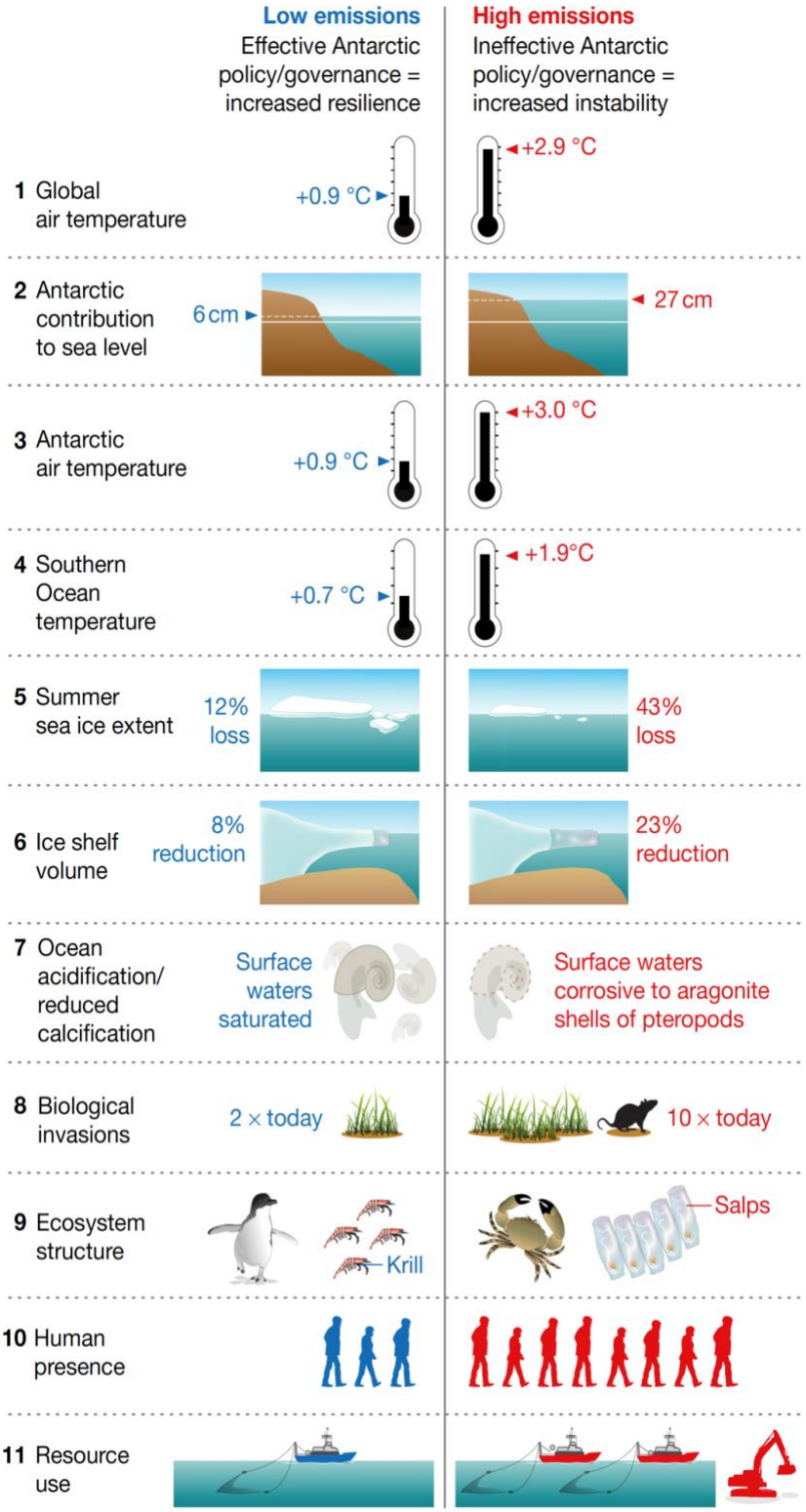


Figure 1.5. Predicted stressors acting on Antarctica and the Southern Ocean marine environment in the year 2070 under low anthropogenic CO_2 emissions (high level of action taken to reduce emissions) and high anthropogenic CO_2 emissions (low level of action taken to reduce emissions). Figure derived from Rintoul *et al.* (2018).

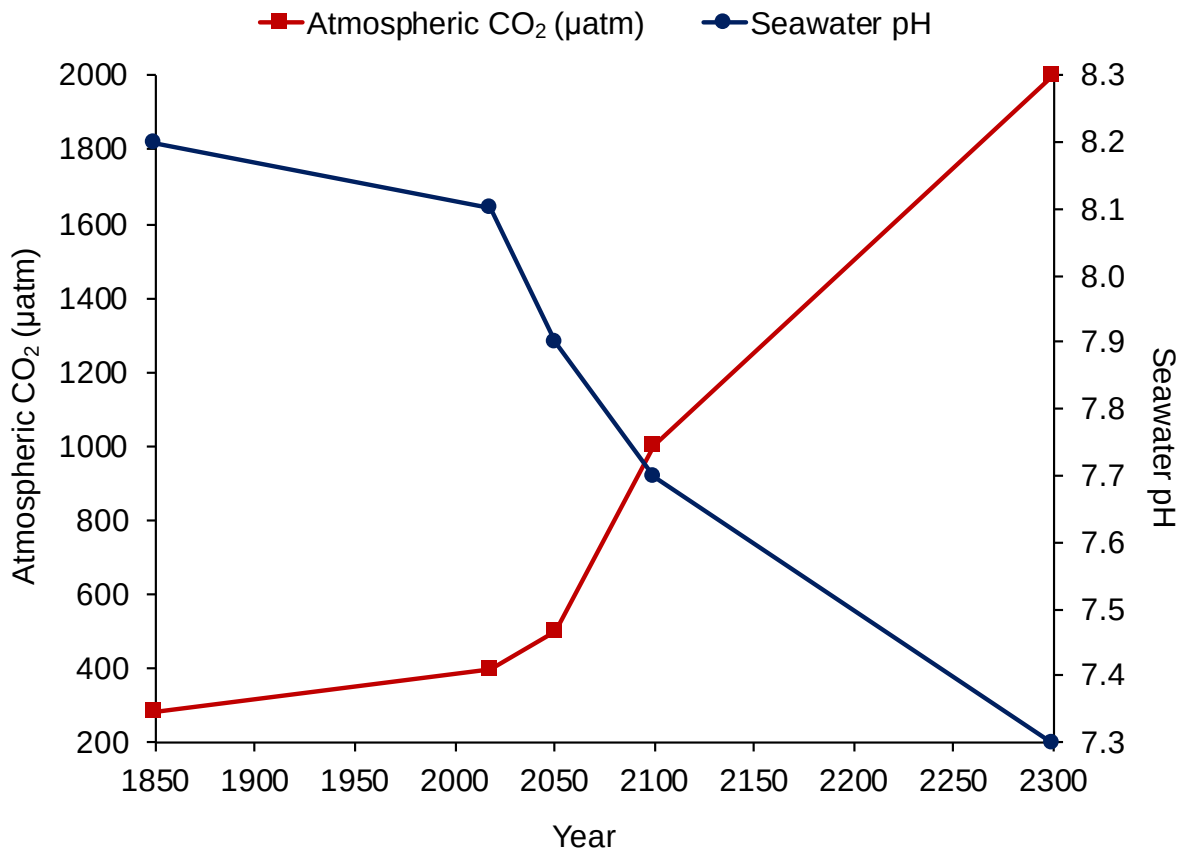


Figure 1.6. The relationship between average global atmospheric CO_2 (µatm) and seawater pH. Current and predicted future values of atmospheric CO_2 and seawater pH are based on data derived from Caldeira & Wickett (2003), Feely *et al.* (2009) and Kawaguchi *et al.* (2013).

Ocean acidification is known to affect a range of processes in marine organisms including growth, calcification, survival, behavior, extra- and intra-cellular pH regulation, and reproduction (Pörtner *et al.* 2014; Gattuso *et al.* 2015; Hurd *et al.* 2018). Meta-analyses of ocean acidification studies have found that although effects are usually negative, they can also be neutral or positive (Hendriks *et al.* 2010; Kroeker *et al.* 2010; Wittmann & Pörtner 2013). Ocean acidification is predicted to be most severe at high latitude oceans as the Southern Ocean is a major CO_2 sink (Fabry *et al.* 2009; Landschützer *et al.* 2015; Rintoul *et al.* 2018).

1.4. Aims and Structure of the Thesis

The overarching aim of this research was to provide a synthesis on the biochemistry and inferred diet of krill in the present day, and to predict how krill may fare in a future high CO_2 world. Specifically, the aims were:

I. To use fatty acids as dietary biomarkers to investigate seasonal and interannual fluctuations in krill diet. Chapter 2 investigates the fatty acid content and composition of krill at an unprecedented resolution using samples obtained from the krill fishery. This study is the first detailed examination of fatty acids in krill across all seasons and for three consecutive years. We also aimed to demonstrate the benefits of using fishing vessels to collect scientific samples.

II. To examine the fatty acid composition of the separate lipid classes and the sterol composition of krill. These results (presented in Chapter 3) aimed to establish for the first time whether sterol composition and the fatty acid composition of the lipid classes varied seasonally.

III. To investigate the effects of anthropogenic climate change (specifically, ocean acidification) on the physiology and biochemistry of adult krill during a laboratory study. To our knowledge, this is the first study to examine how adult krill respond to ocean acidification over a long-term period (one year). Results of this research are presented in Chapter 4.

IV. To understand how future ocean change may affect krill fatty acid content and composition. We primarily examined whether elevated seawater CO_2 levels may change the fatty acid composition of krill, as this may not only affect krill, but have potential flow-on effects to higher trophic levels. These results are presented in Chapter 5.

In Chapter 6 the results from the above chapters are synthesized into a General Discussion on krill lipid composition in the present day, and possible changes that will occur in krill populations into the future.

2 Seasonal and interannual variations in the fatty acid composition of adult *Euphausia superba* Dana, 1850 (Euphausiacea) samples derived from the Scotia Sea krill fishery

This chapter has been published:

Ericson JA, Hellessey N, Nichols PD, Kawaguchi S, Nicol S, Hoem N, Virtue P (2018). Seasonal and interannual variations in the fatty acid composition of adult *Euphausia superba* Dana, 1850 (Euphausiacea) samples derived from the Scotia Sea krill fishery. *Journal of Crustacean Biology*, **38** (6): 656 – 661. doi: 10.1093/jcbiol/ruy032.

2.1. Abstract

The fatty acid content and composition of the Antarctic krill *Euphausia superba* Dana, 1850 were investigated using samples collected by a commercial fishing vessel. This dataset allowed comparison between seasons, years (2013 – 2016), and different fishing locations. Quantities of omega 3 fatty acids 20:5n-3 and 22:6n-3 (mg g^{-1} dry mass; DM) were highest in autumn and decreased through winter to reach a spring low. Quantities of the flagellate marker 18:4n-3 and diatom marker 16:1n-7c were variable and did not display the same seasonal fluctuations. In summer, krill had high percentages (% total fatty acids) of 20:5n-3 and 22:6n-3, total PUFA, and low 18:1n-9c/18:1n-7c ratios, indicating a more herbivorous diet. Krill became more omnivorous from autumn to spring, indicated by increasing ratios of 18:1n-9c/18:1n-7c and percentages of Σ 20:1 + 22:1 isomers. Bacterial fatty acids (Σ C_{15} + C_{17} + C_{19} isomers) were minor components year-round (0.9 – 1.8 %). Seasonal levels of herbivory and omnivory differed between years, and levels of specific fatty acid ratios differed between fishing locations. The fatty acid 18:4n-3 was a major driver of variability in krill fatty acid composition, with no obvious seasonal driver. This is the first study to report krill fatty acid data during all four seasons over consecutive years. This large-scale study highlights the value of using fisheries samples to examine seasonal and annual fluctuations in krill diet and condition.

2.2. Introduction

Euphausia superba Dana, 1850, the Antarctic krill (hereafter 'krill'), is one of the most abundant species on Earth (Gigliotti *et al.* 2011) and a keystone species in the Southern Ocean due to its pivotal role in the food web (Loeb *et al.* 1997; Saba *et al.* 2014). Krill are the main food source for Southern Ocean birds, fishes, and mammals, comprising up to 90% of the diet of these predators (Phleger *et al.* 2002). The biomass of krill is estimated to be 60 – 420 million metric tons (Nicol *et al.* 2000; Atkinson *et al.* 2009). Up to 30% of this krill biomass is lipid (Hagen *et al.* 2001). Krill are high in omega 3 fatty acids which have high nutritional value for their predators due to their important role in regulating the structure of cell membranes (Dalsgaard *et al.* 2003).

Krill are also the target of a commercial fishery and sold as meal for aquaculture or as 'krill oil' capsules for human consumption (Nicol *et al.* 2012). The omega 3 krill oil fishery has the potential to expand (Descamps *et al.* 2016). Any expansion of the krill fishery would require data on the biology and ecology of krill at a variety of spatial and temporal scales to provide information that can be used in ecosystem-based management.

Due to their ecological and commercial importance, the fatty acid content and composition of krill have been extensively researched (Clarke 1980; Virtue *et al.* 1993a, 1996; Phleger *et al.* 2002; Hagen *et al.* 2007; Schmidt *et al.* 2014; Reiss *et al.* 2015). Studies have found that krill lipid levels vary seasonally due to processes such as reproduction and growth, and the quality and availability of food at different times of year.

Specific fatty acids, classes, and ratios have been used as 'biomarkers' to investigate the diet of krill (Cripps & Atkinson 2000; Falk-Petersen *et al.* 2000; Stübing & Hagen 2003) and give an insight into the diet over longer time scales than traditional gut content examination (Stübing *et al.* 2003). The fatty acids 20:5n-3, 16:1n-7c, and 16:4n-1 are biomarkers for

diatoms, whereas 18:4n-3 and 22:6n-3 may be derived from the consumption of flagellates (Falk-Petersen *et al.* 2000; Dalsgaard *et al.* 2003, Schaafsma *et al.* 2017). 18:1n-7c is a product of the elongation of 16:1n-7c and is used as a phytoplankton indicator, whereas 18:1n-9c is derived from carnivorous feeding (Falk-Petersen *et al.* 2000). Some copepods are rich in the 20:1 and 22:1 isomers that can be used as biomarkers for copepod consumption (Falk-Petersen *et al.* 2000; Ju & Harvey 2004).

Most studies on the life history and biochemistry of krill have been conducted using research vessels, which face numerous logistical constraints such as limited research time, high costs, and extensive ice cover in the Antarctic winter. As a result, most of the data available on krill fatty acids have been from a single season (Virtue *et al.* 1996; Cripps *et al.* 1999; Cripps & Atkinson 2000; Atkinson *et al.* 2002; Phleger *et al.* 2002; Ju & Harvey 2004; Schmidt *et al.* 2006; O'Brien *et al.* 2011) or combined from a limited range of seasons collected across different years or regions (Fricke *et al.* 1984; Hagen *et al.* 2001; Schmidt *et al.* 2014; Reiss *et al.* 2015). No studies have continuously collected data on krill fatty acids during all four seasons and for consecutive years. Most data have been collected during the summer months and the paucity of winter data has been highlighted (Atkinson *et al.* 2012).

Commercial fishing operations were originally carried out in summer, but with decreasing sea ice cover in the Antarctic Peninsula region, fishing is now being conducted year-round (Kawaguchi & Nicol 2007). Fishing vessels are increasingly being used in the Antarctic to collect samples and gather information for scientific research (Schmidt *et al.* 2014; Krafft *et al.* 2015; Watkins *et al.* 2016).

Our study utilised krill samples caught regularly by a commercial fishing vessel at a scale not currently feasible using scientific research vessels. We conducted fatty acid analyses on male and female krill caught over three years (2013 to 2016) during all four seasons and

from the major fishing grounds in the Scotia Sea: the Antarctic Peninsula and the South Orkney and South Georgia islands. We investigated spatial and temporal drivers of variation in the fatty acid profiles of krill, and used fatty acid data as a proxy for seasonal changes in krill diet.

2.3. Materials and methods

2.3.1. Sample collection

Krill were caught on board the fishing vessel FV *Saga Sea* (Aker BioMarine, Oslo, Norway) during their December-September fishing seasons from 2013 – 2016. The three fishing seasons (December 2013 – September 2014, December 2014 – September 2015, and December 2015 – September 2016) will hereafter be referred to as the three fishing years 2014, 2015, and 2016, respectively. Because FV *Saga Sea* concluded fishing in mid-September and did not resume until early December every year, spring samples are only from September.

FV *Saga Sea* fished in FAO statistical subareas 48.1 (West Antarctic Peninsula = WAP), 48.2 (South Orkney Islands = SOI), and 48.3 (South Georgia = SG) within the CCAMLR Convention Area (see Hellessey *et al.* (2018) for maps of CCAMLR subareas and sampling locations). The vessel predominantly fished at the West Antarctic Peninsula and the South Orkney Islands during summer and autumn, and South Georgia in winter and spring. Fishing locations varied between years as the vessel moved between the CCAMLR areas when the maximum allowable catch for a subarea was reached (48.1), or when sea ice in a subarea made fishing impossible (48.1 and 48.2) or when it attained production goals for a subarea (48.3).

Krill were fished using a continuous catch system whereby a steady (24 h) stream of live krill is pumped from a mid-water trawl net onto the vessel using the vessel's Eco-

Harvesting. (Aker BioMarine) technology. This method of harvesting ensures that krill specimens remain alive and intact.

A fisheries observer took a random sample of twenty krill individuals per day from the catch (there was no selection by size or maturity stage). The sample was divided into two foil or vacuum-sealed packets, lined up in rows of 10 krill per pack. Krill were frozen immediately in a –20°C freezer on board the vessel for 4 h, then transferred to a –80°C freezer for storage. Sample bags were transported on dry ice to Hobart, Tasmania, where they were again stored in a –80°C freezer until needed for analysis.

At least three adult female and three adult male krill 30 – 60 mm in length were selected from the samples at two-week intervals from December 2013 to September 2016 (391 samples in total). Krill were sexed using a dissecting microscope and weighed (wet mass), and the length of each specimen was measured from the tip of the rostrum to the tip of the uropod using 'Standard 1' measurement (Kirkwood 1982). Krill were kept frozen during this process to prevent degradation. A dry mass was obtained later by multiplying the wet mass by 0.2278 to account for the 77.2% water content in krill (Virtue *et al.* 1993a).

2.3.2 Fatty acid analyses

Whole krill were analysed for fatty acids as the large sample size (N = 391) made it cumbersome to separate out the individual lipid classes (Stübing & Hagen 2003). Specimens were extracted overnight using a modification of the method of Bligh & Dyer (1959), consisting of a methanol:dichloromethane:water ($MeOH/CH_2Cl_2/H_2O$) solvent mixture (20:10:7 mL). Phase separation was carried out the following day by adding 10 ml CH_2Cl_2 and 10 ml saline MilliQ H_2O, giving a final methanol:dichloromethane:water solvent ratio of 1:1:0.85. The lower layer was drained and the total solvent extract was concentrated using

rotary evaporation. The extract was transferred into a pre-weighed 2 ml vial and the solvent was blown down under nitrogen gas to obtain a total lipid extract (TLE) weight. Solvent was added until further procedures were carried out to avoid oxidation.

For fatty acid methyl ester (FAME) preparation, a subsample of the TLE was transferred into a glass test tube fitted with a Teflon-lined screw cap and treated with 3 ml of methylating solution (methanol:dichloromethane: hydrochloric acid, 10:1:1, v:v:v), then heated at 90 – 100 °C for 1 h 15 min. Samples were cooled and 1 ml of H_2O and 1.8 ml of hexane:dichloromethane (4:1, v:v) solution was added to extract the FAME. Samples were then centrifuged for 5 min and the upper layer containing FAME was transferred to a vial, with another 1.8 ml of hexane:dichloromethane then added to the test tube. This process was repeated twice and samples were blown down using nitrogen gas. Samples were made to 1.5 ml with dichloromethane and stored at –20 °C until gas chromatography analysis. Prior to analysis, samples were blown down again using nitrogen gas and 1.5 ml of internal injection standard (23:0 FAME) was added to each vial.

Samples were analysed via gas chromatography using an Agilent Technologies 7890A GC-FID System (Palo Alto, CA, USA) equipped with a non-polar Equity®-1 fused-silica capillary column (15 m length x 0.1 mm internal diameter, 0.1 μm film thickness). Samples (0.2 μl) were injected in splitless mode at an oven temperature of 120 °C with helium as the carrier gas. The oven temperature was raised to 270 °C at a rate of 10 °C per minute, then to 310 °C at 5 °C per minute. Peaks were quantified using Agilent Technologies ChemStation software (Palo Alto, CA, USA) with initial identification based on comparison of retention times with known (Nu Chek Prep mix; http://www.nu-chekprep.com) and fully characterised laboratory (tuna oil) standards. Fatty acid peaks were expressed as a percentage of the total fatty acid area.

Confirmation of component identification was performed by GC-MS of selected samples and was carried out on a Thermo Scientific (Waltham, MA, USA) 1310 GC coupled with a TSQ triple quadruple. Samples were injected using a Tripleplus RSH (Waltham, MA, USA) auto sampler using a non-polar HP-5 Ultra 2 bonded-phase column (50 m length x 0.32 mm internal diameter x 0.17 μm film thickness). The HP-5 column was of similar polarity to the column used for GC analyses. The initial oven temperature of 45 ℃ was held for 1 min, followed by temperature programming at 30 ℃ per minute to 140 ℃, then at 3 ℃ per minute to 310 ℃, where it was held for 12 min. Helium was used as the carrier gas. Mass-spectrometer operating conditions were as follows: electron impact energy 70 eV; emission current 250 μamp, transfer line 310 ℃; source temperature 240 ℃; scan rate 0.8 scan/sec and mass range 40 – 650 Da. Mass spectra were acquired and processed with Thermo Scientific XcaliburTM software (Waltham, MA, USA). Identification and quantification of peaks was conducted using the same standards as GC-FID analysis.

2.3.3. Statistical analyses

Fatty acid quantitative and percentage data for each season were analysed in the RStudio (www.rstudio.com) statistics package (version 0.99.893) using two-way ANOVA, with sex and year as factors. Specific ratios and percentages of fatty acid biomarkers were also analysed using two-way ANOVA with location and year as factors. Two-way ANOVA analyses with season and location as factors were not possible, as data for all seasons were not available for all fishing locations. Type 3 Sums of Squares (SS) were used for statistical analyses when data were unbalanced and Type 1 SS analyses were not appropriate. Data were log- or square-root transformed when they did not meet assumptions of normality. Tukey post-hoc comparisons were used to identify significant differences between factor levels. Data tables are expressed as mean ± standard deviation. For all analyses, α was set at 0.05.

Principal component analyses (PCA) were performed in PRIMER 6 (http://www.primer-e.com) to investigate similarities and differences between groups of fatty acids and identify those that explained most of the variability in the data set. A correlation based PCA (Pearson correlation) was used due to large differences in variances between the fatty acids. Data were transformed using a log (x+1) transformation before analysis, to reduce the influence of fatty acids that had large percentages.

2.4. Results

The mean body length of krill was 46.0 mm (± 4.8), the mean wet mass was 0.72 g (± 0.2) and mean dry mass was 0.16 g (± 0.05). Fifty-nine fatty acids were identified in the krill samples (N = 391). The major fatty acids (as mg g^{-1} DM) were the saturated fatty acids (SFA) 14:0 and 16:0, monounsaturated fatty acids (MUFA) 16:1n-7c, 18:1n-7c, and 18:1n-9c and the essential omega 3 polyunsaturated fatty acids (PUFA) 20:5n-3 and 22:6n-3 (eicosapentaenoic acid, EPA, and docosahexaenoic acid, DHA, respectively).

The total fatty acid percentage composition of female and male krill in all fishing years and seasons is summarised in Appendix I. Individual fatty acids and ratios used as biomarkers for krill diet are listed in Table 2.1 (diatom markers 16:1n-7c and 20:5n-3; flagellate markers 18:4n-3 and 22:6n-3; 18:1n-9c/18:1n-7c (carnivory/phytoplankton) ratio; Σ 20:1 + 22:1 isomers (copepod markers); Σ C_{15} + C_{17} + C_{19} isomers (bacterial markers). Percentages of these fatty acids and fatty acid ratios were used to examine fine scale seasonal and interannual differences between krill. Percentages of PUFA, MUFA and SFA in krill in different seasons and years are also shown in Appendix II.

Most of the fatty acid percentage and ratio data were not significantly different (p > 0.05) between males and females, so data from both sexes were combined. The limited number of sex differences observed are summarised in Table 2.2. These sex differences were

consistently observed for each of the years sampled (see also Appendix I).

Table 2.1. Interannual differences in the biomarker fatty acid composition (% of total fatty acids, mean ± SD) and fatty acid ratios of *Euphausia superba* collected in summer, autumn, winter, and spring during the different fishing years. Significant differences between years are denoted by cells that do not share lower case letters, and probability (*p*) values are stated for significant differences. The highest *p* value is stated when there is more than one significant difference between years; NS, no significant difference ($p > 0.05$).

Summer				
Fatty Acid	**2014**	**2015**	**2016**	***p* value**
16:1n-7c	4.5 ± 2.6 ^{a}	6.9 ± 2.0 ^{b}	6.9 ± 1.7 ^{b}	< 0.001
18:4n-3	2.6 ± 1.5 ^{a}	3.4 ± 1.1 ^{b}	3.6 ± 1.3 ^{b}	< 0.002
20:5n-3	20.3 ± 4.5 ^{a}	17.9 ± 1.9 ^{bc}	19.1 ± 3.3 ^{ac}	< 0.008
22:6n-3	17.4 ± 7.5 ^{a}	9.9 ± 3.2 ^{b}	9.4 ± 3.7 ^{b}	< 0.001
18:1n-9c / 18:1n7c	1.4 ± 0.3 ^{a}	1.8 ± 0.5 ^{b}	1.9 ± 0.5 ^{b}	< 0.001
Σ 20:1 + 22:1 isomers	1.8 ± 0.7 ^{a}	2.1 ± 0.5 ^{b}	2.1 ± 0.5 ^{b}	< 0.062
Σ C_{15} +C_{17} + C_{19} isomers	1.6 ± 0.6 ^{a}	1.4 ± 0.6 ^{ac}	1.1 ± 0.3 ^{c}	< 0.001
Autumn				
Fatty Acid	**2014**	**2015**	**2016**	***p* value**
16:1n-7c	8.6 ± 1.8 ^{a}	5.4 ± 1.6 ^{b}	9.3 ± 1.3 ^{a}	< 0.001
18:4n-3	2.2 ± 1.3 ^{a}	5.3 ± 1.9 ^{b}	2.2 ± 0.7 ^{a}	< 0.001
20:5n-3	15.3 ± 2.1 ^{a}	17.3 ± 1.6 ^{b}	15.9 ± 2.3 ^{a}	< 0.005
22:6n-3	7.3 ± 1.9 ^{a}	9.5 ± 1.6 ^{b}	5.6 ± 1.8 ^{c}	< 0.001
18:1n-9c / 18:1n7c	1.8 ± 0.3 ^{a}	1.6 ± 0.3 ^{b}	2.3 ± 0.4 ^{c}	< 0.011
Σ 20:1 + 22:1 isomers	2.8 ± 0.6 ^{a}	2.2 ± 0.4 ^{b}	2.8 ± 0.5 ^{a}	< 0.001
Σ C_{15} +C_{17} + C_{19} isomers	1.4 ± 0.3 ^{a}	1.5 ± 0.4 ^{a}	0.9 ± 0.4 ^{b}	< 0.001
Winter				
Fatty Acid	**2014**	**2015**	**2016**	***p* value**
16:1n-7c	6.7 ± 1.2 ^{a}	8.0 ± 1.1 ^{b}	9.5 ± 1.0 ^{c}	< 0.001
18:4n-3	3.9 ± 1.8 ^{a}	1.9 ± 0.8 ^{b}	1.4 ± 0.6 ^{c}	< 0.003
20:5n-3	16.1 ± 1.6 ^{a}	16.0 ± 1.7 ^{a}	13.8 ± 1.0 ^{b}	< 0.001
22:6n-3	9.4 ± 1.7 ^{a}	8.3 ± 1.3 ^{b}	6.3 ± 1.1 ^{c}	< 0.003
18:1n-9c / 18:1n-7c	1.9 ± 0.3 ^{a}	2.0 ± 0.2 ^{a}	2.0 ± 0.4 ^{a}	NS
Σ 20:1 + 22:1 isomers	2.2 ± 0.5 ^{a}	2.9 ± 0.5 ^{b}	2.8 ± 0.5 ^{b}	< 0.001
Σ C_{15} +C_{17} + C_{19} isomers	1.8 ± 0.3 ^{a}	1.4 ± 0.3 ^{b}	1.4 ± 0.3 ^{b}	<0.001
Spring				
Fatty Acid	**2014**	**2015**	**2016**	***p* value**
16:1n-7c	6.8 ± 1.1^{a}	7.0 ± 0.7 ^{a}	7.9 ± 0.9 ^{b}	< 0.061
18:4n-3	1.6 ± 0.8 ^{a}	1.1 ± 0.2 ^{ab}	1.0 ± 0.3 ^{b}	< 0.012
20:5n-3	17.1 ± 1.2 ^{a}	17.7 ± 2.2 ^{a}	15.2 ± 1.5 ^{b}	< 0.051
22:6n-3	10.6 ± 1.4 ^{a}	9.9 ± 0.7 ^{a}	8.7 ± 15 ^{b}	< 0.059
18:1n-9c / 18:1n-7c	2.0 ± 0.2 ^{a}	2.1 ± 0.3 ^{a}	2.2 ± 0.3 ^{a}	NS
Σ 20:1 + 22:1 isomers	2.9 ± 0.6 ^{a}	2.7 ± 0.6 ^{a}	2.8 ± 0.4 ^{a}	NS
Σ C_{15} +C_{17} + C_{19} isomers	1.5 ± 0.2 ^{a}	1.4 ± 0.3 ^{a}	1.4 ± 0.1 ^{a}	NS

Table 2.2. Significant differences ($p < 0.05$) between male and female *Euphausia superba* in the composition (% of total fatty acids) of biomarker fatty acids and ratios. Differences are shown for each season. No Sex*Fishing Year interactions were found, therefore these were consistent trends seen in each fishing year; NS, no significant difference ($p > 0.05$).

Season	Fatty Acid	Difference	*p* value
Summer	No differences	NS	NS
Autumn	18:1n-9c/18:1n-7c	males > females	0.008
Winter	18:4n-3	males > females	0.010
	16:1n-7c	females > males	0.025
Spring	20:5n-3	males > females	0.014

2.4.1. Seasonal and interannual variations in quantities of selected fatty acids

Quantities of 20:5n-3 and 22:6n-3 (as mg g^{-1} DM) in krill were highest in autumn, decreasing through winter to reach a spring low (Figure 2.1A, B), displaying the same seasonal variation as total lipid content (see Hellessey *et al.* 2018). Significant interannual differences in fatty acid quantities were observed in autumn. Average quantities of 20:5n-3 were significantly lower in autumn 2014 (27.1 ± 11.1) and autumn 2015 (30.2 ± 10.8) compared with autumn 2016 (53.4 ± 21.4) ($p < 0.001$). Average quantities of 22:6n-3 were significantly lower in the autumn of 2014 (12.5 ± 4.8) compared with 2015 (16.5 ± 6.5) and 2016 (17.8 ± 5.6) ($p < 0.01$). When data from autumn of all years were combined, quantities of 20:5n-3 and 22:6n-3 in krill did not differ between the West Antarctic Peninsula (WAP) and South Orkney Islands (SOI) fishing locations ($p = 0.429$ and $p = 0.147$, respectively).

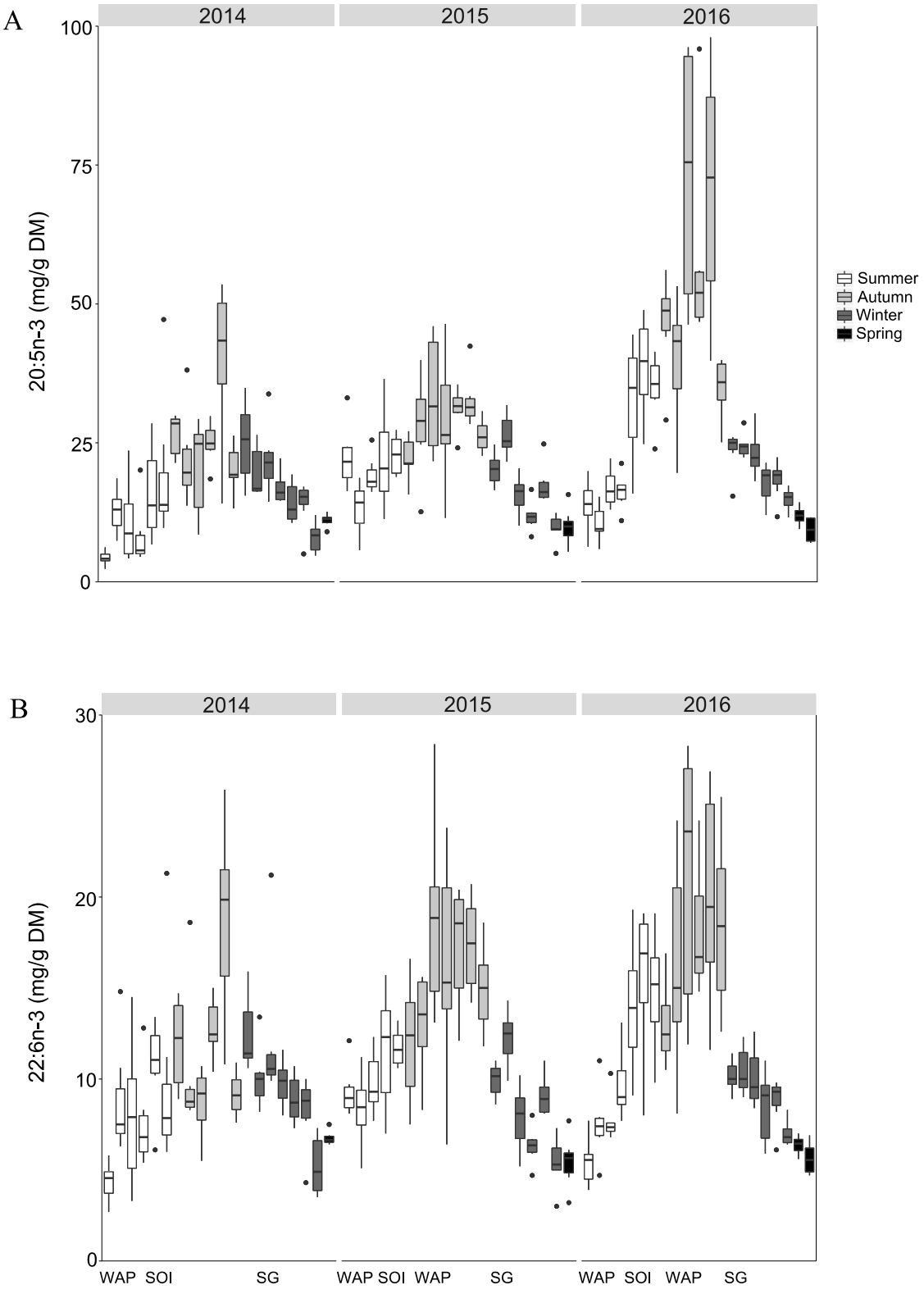


Figure 2.1. Quantities of eicosapentaenoic acid (EPA; 20:5n-3 mg g $^{-1}$ DM) (**A**) and docosahexaenoic acid (DHA; 22:6n-3 mg g $^{-1}$ DM) (**B**) in *Euphausia superba* in summer, autumn, winter, and spring of each fishing year (2014, 2015, and 2016). Individual boxes represent the median, upper, and lower quartiles and minimum and maximum values (≤ 1.5 times the interquartile range) for n = 6 krill (3 males and 3 females) sampled at two-week intervals within each season. Shaded black dots denote outliers > 1.5 times the interquartile range. Fishing locations are shown on the x axis (WAP, West Antarctic Peninsula; SOI, South Orkney Islands; SG, South Georgia).

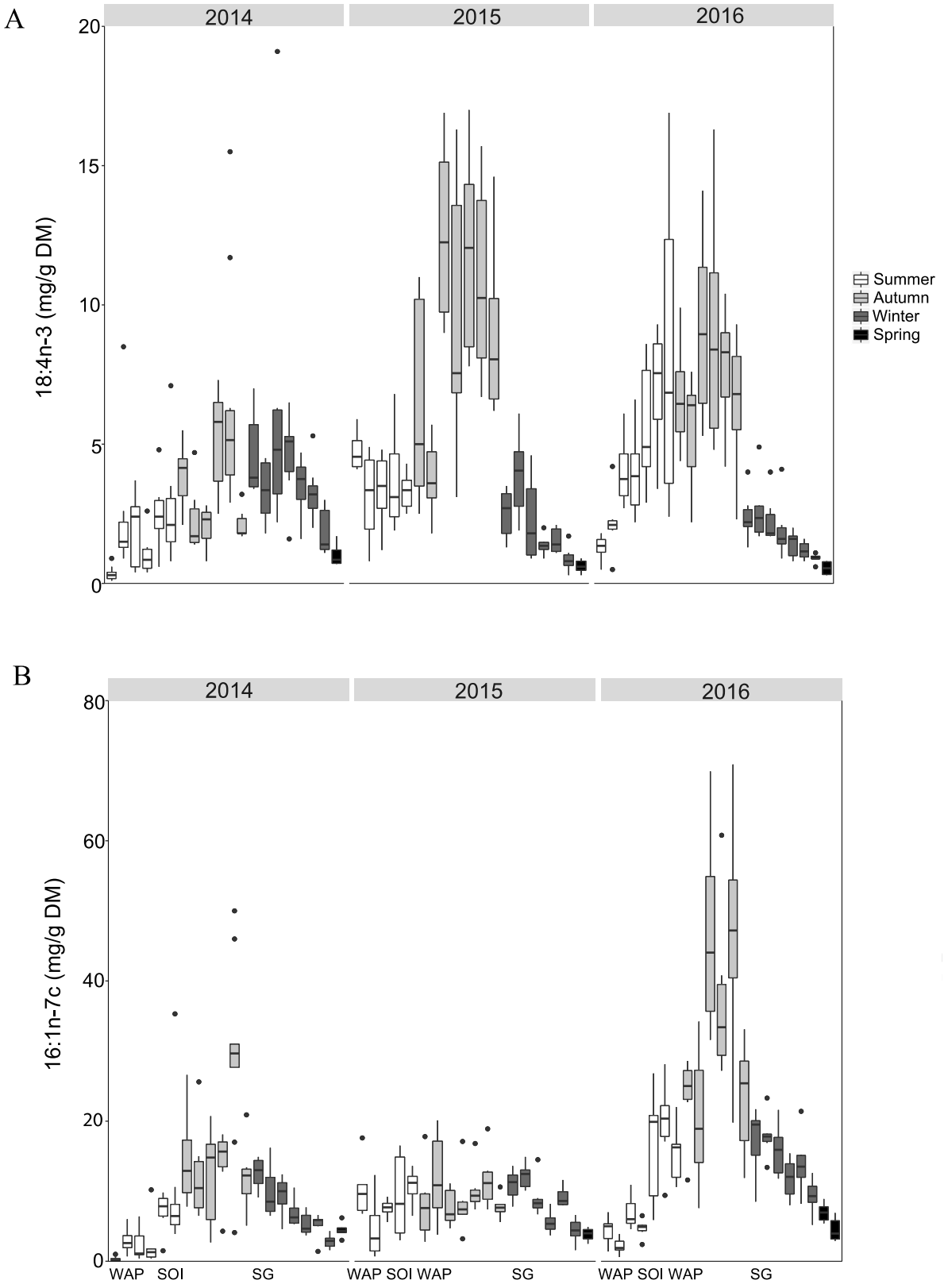


Figure 2.2. Quantities of 18:4n-3 (mg g $^{-1}$ DM) (**A**) and 16:1n-7c (mg g $^{-1}$ DM) (**B**) in *Euphausia superba* in summer, autumn, winter, and spring of each fishing year (2014, 2015, and 2016). Individual boxes represent the median, upper, and lower quartiles and minimum and maximum values (≤ 1.5 times the interquartile range) for *n* = 6 krill (3 males and 3 females) sampled at two-week intervals within each season. Shaded black dots denote outliers > 1.5 times the interquartile range. Fishing locations are shown on the x axis (WAP, West Antarctic Peninsula; SOI, South Orkney Islands; SG, South Georgia).

Quantities of the flagellate marker 18:4n-3 were less predictable and did not exhibit the same strong seasonal pattern as 20:5n-3 and 22:6n-3. Average quantities of 18:4n-3 (mg g^{-1} DM) were highest in autumn of 2015 (9.6 ± 5.2) and 2016 (7.4 ± 3.0) compared with 2014 (3.9 ± 2.8) ($p < 0.05$) and had declined markedly by spring (< 1.0 for all years) (Figure 2.2A). Average quantities of 18:4n-3 through 2014 were lower than in 2015 and 2016 and peaked in winter (4.0 ± 2.8). A number of individuals in 2014 had larger than expected quantities of 18:4n-3.

Quantities of the diatom marker 16:1n-7c also showed high variability between years and followed a similar pattern to 20:5n- 3, with highest average concentrations in the autumn 2016 (32.7 ± 15.9) (Figure 2.2B). This quantity was 1.9 – 3.5 times higher than in autumn 2014 (16.9 ± 10.7) and 2015 (9.4 ± 4.4). When data from autumn of all years were combined, quantities of 18:4n-3 and 16:1n-7c in krill did not differ between the WAP and SOI fishing locations ($p = 0.944$ and $p = 0.108$, respectively).

2.4.2. Seasonal and interannual variations in the percentage composition of fatty acids

Summer

Some consistent patterns in the fatty acid composition of krill during summer were observed in all three years (Table 2.1). Krill had high percentages of 20:5n-3, 22:6n-3 and total PUFA, moderate percentages of 16:1n-7c, Σ 20:1 + 22:1 isomers and Σ C_{15} + C_{17} + C_{19} isomers, and low 18:1n-9c/18:1n-7c ratios compared with other seasons. Percentages of 18:4n-3 were less predictable with high percentages in the 2016 summer and high variability between years. Krill in the 2015 and 2016 summers had similar fatty acid profiles for the range of fatty acids and ratios listed in Table 2.1. The fatty acid profile of krill in the 2014 summer was anomalous due to high percentages of 22:6n-3 that dominated the proportion of fatty acids in krill that summer.

Autumn

Krill caught in autumn were lipid rich (Hellessey *et al.* 2018) and had the highest quantities of the major fatty acids during this season. They had moderate to high percentages of 16:1n-7c, Σ 20:1 + 22:1 isomers and ratios of 18:1n-9c/18:1n-7c compared with other seasons. Percentages of 20:5n-3 and 22:6n-3 in krill were generally lower than in summer, with the exception of 2015, where they were equal to summer. Bacterial-marker fatty acids made up a small but consistent (0.9 – 1.5%) part of the profile, at levels similar to summer. Krill sampled in autumn 2014 and 2016 were most similar to each other for the fatty acids listed in Table 2.1. The fatty acid profiles of krill sampled in autumn 2015 were anomalous due to elevated percentages of omega 3 fatty acids 18:4n-3, 20:5n-3, and 22:6n-3, and total PUFA, and lower percentages of 16:1n-7c and Σ 20:1 + 22:1 isomers compared with the other two years.

Winter

Percentages of Σ 20:1 + 22:1 isomers, bacterial markers, and SFA in krill were generally higher in winter. Percentages of 20:5n-3 and 22:6n-3 were low in winter compared with summer and autumn, and particularly low in 2016. Percentages of 18:4n-3 in krill were elevated in winter of 2014, whereas 16:1n-7c was generally high in winter of all years (Table 2.1). Percentages of 16:1n-7c, 18:4n-3, and 22:6n-3 in krill differed significantly between 2014, 2015, and 2016 (Table 2.1). Fatty acid profiles in 2014 were flagellate-marker dominant (higher 18:4n-3 and 22:6n-3), whereas 2016 krill were higher in the diatom marker 16:1n-7c (Table 2.1). Krill sampled in the 2015 winter had moderate percentages of both flagellate and diatom markers. Krill caught in the 2014 winter had less Σ 20:1 + 22:1 isomers and more bacterial markers than subsequent years.

Spring

Krill caught in spring were low in total lipids compared with the other seasons (see Hellessey

et al. 2018). They had consistently high 18:1n-9c/18:1n-7c ratios and Σ 20:1 + 22:1 isomers and had similar fatty acid profiles to winter krill. Krill in spring were particularly low in 18:4n-3 compared with other seasons. Spring fatty acid composition was similar across all years with the exception of higher percentages of 16:1n-7c and lower percentages of omega 3 fatty acids in 2016 compared with the other years (Table 2.1).

2.4.3. Principal component analyses of fatty acid composition (%) of krill within fishing years

Principal component analyses were performed on fatty acid percentages of krill to examine the major drivers of variability within fishing years. Only the 15 fatty acids accounting for > 0.5% of the total fatty acid profile (fatty acids listed in Appendix I) were included in the analysis.

The first two principal components (PC1 and PC2) accounted for 84.4% of the variation in the data in 2014 (see Appendix III for PCA outputs). PC1 clearly separated summer krill from the other seasons (Figure 2.3A). This was attributed to high percentages of 22:6n-3, 20:5n-3, and 18:3n-3 and low percentages of 14:0, 16:1n-7c, and 22:1n-9c, compared with the opposite pattern seen in winter, autumn, and spring krill. The fatty acid 18:4n-3 had the highest loading on PC2. Winter krill were high in 18:4n-3, 18:3n-3, 14:0, and 16:4n-1, and contained lower percentages of 18:1n-7c, 18:1n-9c, and 20:1n-9c than autumn and spring krill with the opposite profiles.

Fatty acid profiles displayed a different pattern in 2015. The first two principal components (PC1 and PC2) accounted for 78.6% of the variation in the data (Appendix III). PC1 separated summer and autumn krill with higher percentages of flagellate markers 18:4n-3 and 18:3n-3 from winter and spring krill more dominant in the diatom marker 16:1n-7c, and 18:1n-9c, 20:1n-9c, and 22:1n-9c (Figure 2.3B). The fatty acid 16:1n-7c was high in winter

krill, and it was a major driver separating winter krill from krill collected in the other seasons. The fatty acid 16:4n-1 had the highest loading on PC2 due to the low percentages found in spring compared with the other seasons. PC2 also separated winter krill with higher percentages of 16:1n-7c, 18:0, 20:1n-9c, and 21:5n-3 from summer and autumn krill which were higher in 22:6n-3 and 18:2n-6. This may be explained by the three summer outliers with very high 22:6n-3 percentages. It also identifies autumn krill as having high percentages of 18:4n-3 compared with other seasons.

The first two principal components (PC1 and PC2) accounted for 76.6% of the variation (Appendix III) in 2016. PC1 separated summer krill with higher than average percentages of 18:4n-3, 18:3n-3, 20:5n-3, 22:6n-3, and 16:4n-1 from krill in the other seasons that were higher in 14:0, 16:1n-7c, 18:1n-9c, 20:1n-9c, and 22:1n-9c (Figure 2.3C). PC2 was dominated by summer krill higher in 18:4n-3, 16:4n-1, and 21:5n-3 compared with winter and spring krill with elevated percentages of 18:2n-6 and spring krill higher in 22:6n-3.

The flagellate marker 18:4n-3 had the largest influence on the variability within years (with the largest number of high loadings in Appendix III) but was not correlated with any particular season.

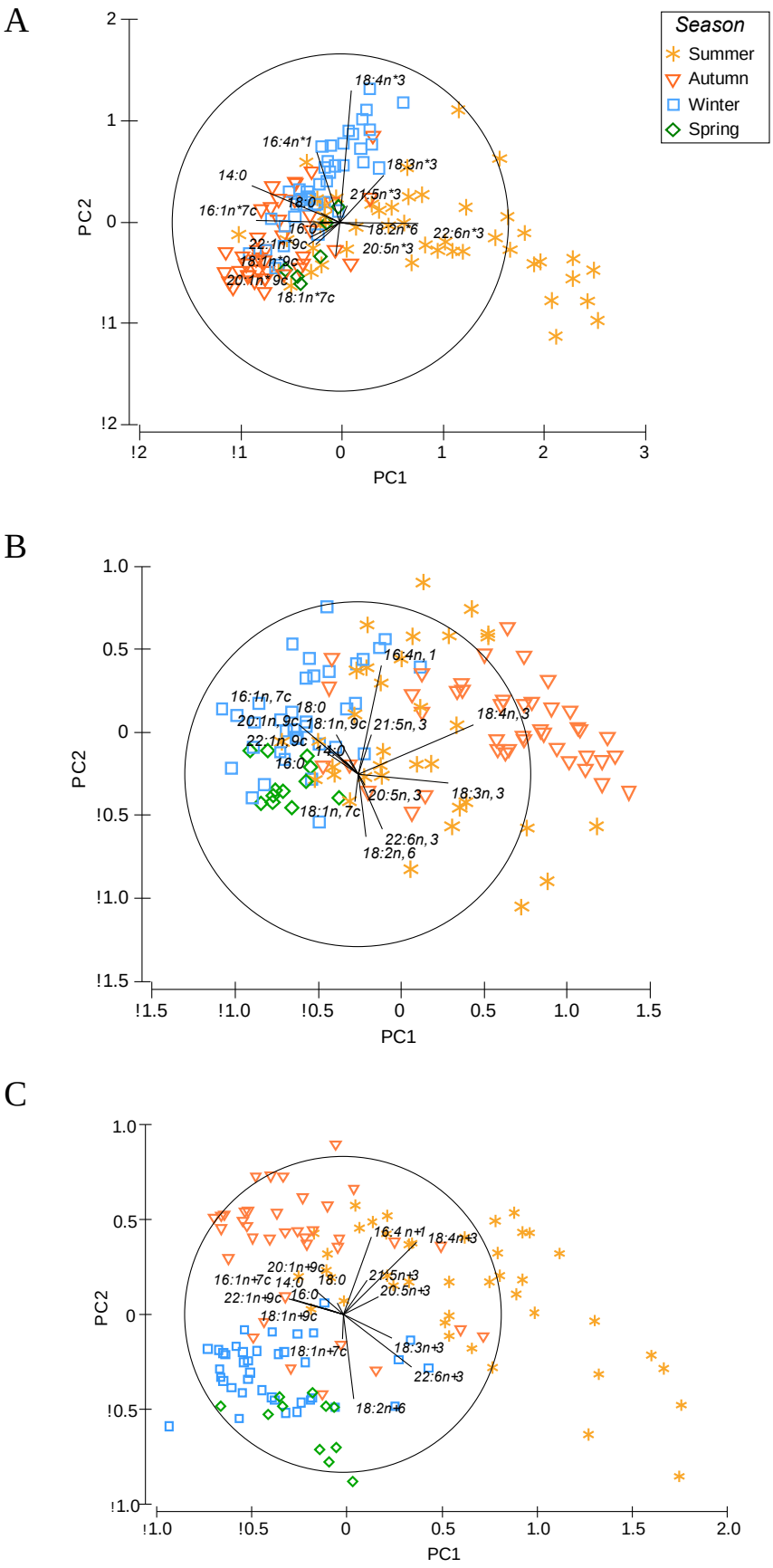


Figure 2.3. Principal component analysis of fatty acid percentage composition of *Euphausia superba* in 2014 (**A**), 2015 (**B**), and 2016 (**C**).

2.4.4. Effect of fishing location on fatty acid biomarkers

Levels of four fatty acid biomarkers (20:5n-3/22:6n-3, 16:1n-7c/18:4n-3, 18:1n9-c/18:1n-7c ratios, and Σ 20:1 + 22:1 isomers) were compared between fishing locations and years to examine the effect of location on krill diet (Figure 2.4).

Strong interannual variations in fatty acid biomarker ratios were evident for all locations, and there were no consistent trends observed across all three years for any of the biomarkers examined (Location*Fishing Year $p < 0.001$ for all analyses). Ratios of the diatom/flagellate biomarker 20:5n-3/22:6n-3 were lower at the WAP in 2014 (1.2 ± 0.4, $p < 0.001$), and higher at the WAP in 2016 (2.8 ± 0.9, $p < 0.001$) compared with other years and locations (Figure 2.4). Ratios of the diatom/flagellate biomarker 16:1n-7c/18:4n-3 were low at the WAP in 2014 (1.4 ± 0.9) and 2015 (1.4 ± 1.0), and significantly higher at SG in 2016 (8.2 ± 3.3, $p < 0.001$) (Figure 2.4). The carnivory/herbivory ratio 18:1n-9c/18:1n- 7c and Σ 20:1 + 22:1 isomers (copepod indicator) were lower at the WAP in 2014 than other years and locations (1.3 ± 0.3 and 1.5 ± 0.3 respectively, $p < 0.001$) (Figure 2.4).

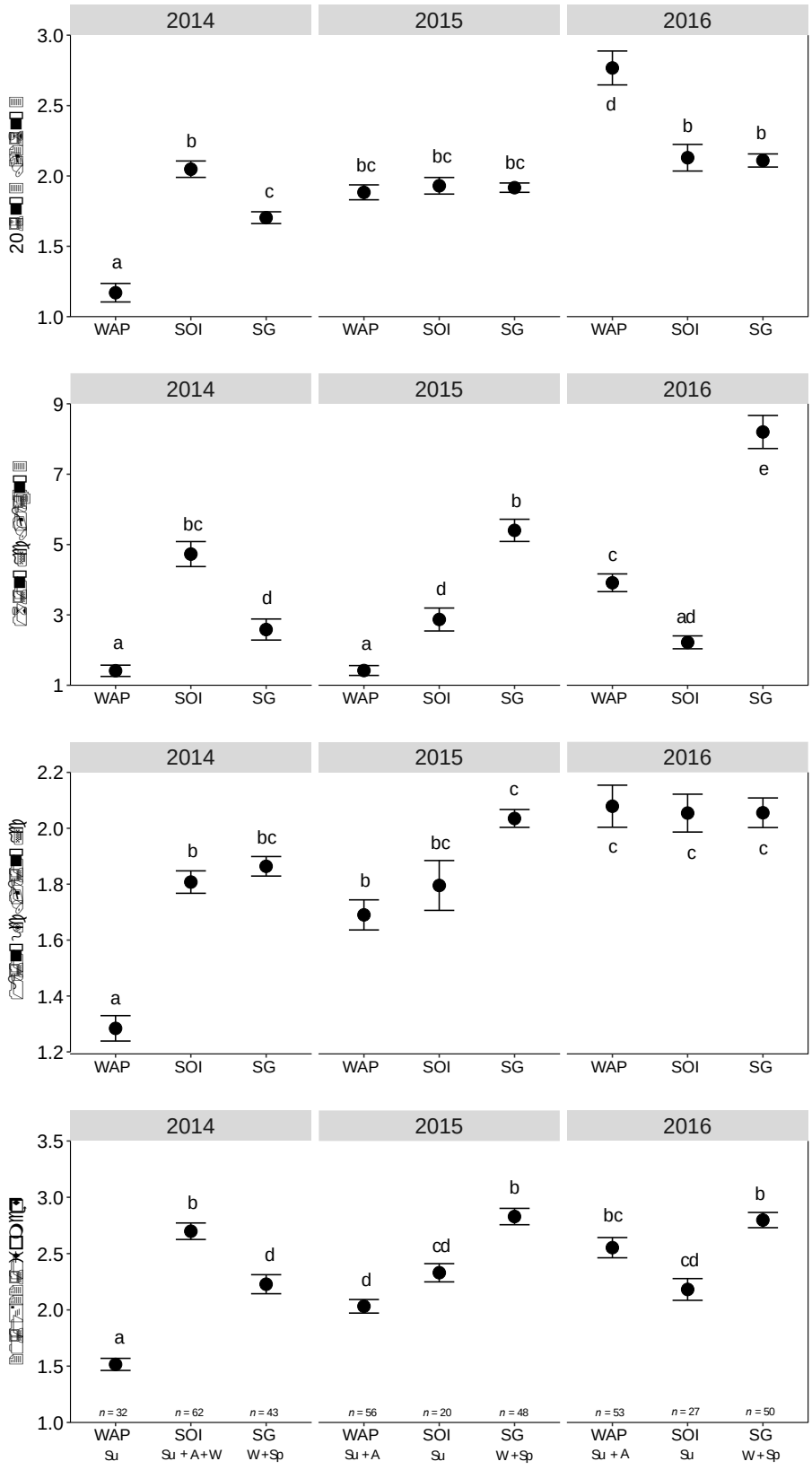


Figure 2.4. Ratios of 20:5n-3/22:6n-3 and 16:1n-7c/18:4n-3 (diatom/flagellate ratios), 18:1n-9c/18:1n-7c (carnivory/herbivory ratio), and Σ 20:1 + 22:1 isomers (copepod dietary indicator) in *Euphausia superba* in different locations and years (mean ± SE). WAP, West Antarctic Peninsula, SOI, South Orkney Islands, SG, South Georgia. The sample size (*n*) for each location and year combination is provided. The seasons in which krill were sampled in each location and year are also provided (Su, summer; A, autumn; W, winter; Sp, spring). Significant differences ($p < 0.05$) between locations and years are denoted by points that do not share lower case letters.

2.5. Discussion

Strong seasonal trends in the content and composition of krill fatty acids were observed in the regularly collected samples from the krill fishery. There were also notable variations between years and fishing locations, and minor differences between sexes.

Our time series is the most comprehensive data set on krill fatty acids so far available. The fatty acid composition of krill was, however, similar to previous studies that have been more limited in sampling scope (Hagen *et al.* 2001; Ju & Harvey 2004; Schmidt & Atkinson 2016). Phytoplankton, copepod, and bacterial biomarkers were present in the fatty acid profiles of krill year-round, although krill were generally more herbivorous in summer, and became more omnivorous as the seasons progressed through to the following spring. Earlier studies on the diet of krill suggested that they were solely herbivorous (Marr 1962), but more recent studies agree that krill can feed on bacteria (Virtue *et al.* 1997, Schmidt *et al.* 2006), display some degree of omnivory year-round (Schmidt *et al.* 2014), and continue to feed throughout the winter albeit at lower levels compared with summer (Atkinson *et al.* 2002).

Percentages of a small number of fatty acids differed between male and female krill in our study, but there were no significant sex differences for most of the fatty acids analysed. Other studies have also found varied results with respect to differences in fatty acids between sexes. In summer, for example, males can have higher percentages of 20:5n-3 and 22:6n-3 than females (Virtue *et al.* 1996), or lower PUFA than females (Phleger *et al.* 1998), whereas other studies have not found consistent differences in fatty acid composition between sexes (Hagen *et al.* 2001; Schmidt *et al.* 2006). Variability in lipid content and composition can also be very high between sites and mask differences between sexes (Pond *et al.* 1995; Schmidt *et al.* 2006), and diet is thought to be a stronger driver of fatty acid composition than maturity or sex

(Cripps *et al.* 1999). A further use of the fishery-derived samples could be to examine in detail the relationship between maturity stage and sex and lipid profiles throughout the year.

The strong seasonal cycle seen in 20:5n-3 and 22:6n-3 quantities, with an increase through summer to a peak in autumn, and then a low in spring, has been documented in previous studies (Hagen *et al.* 2001; Schmidt *et al.* 2014). This seasonal cycle is linked to the abundance of food and the total lipid content of krill, as krill build up lipid stores over the late spring, summer, and autumn when food is abundant and decrease their feeding during winter and early spring (Meyer *et al.* 2010, Schmidt *et al.* 2014; Schmidt & Atkinson 2016).

The strong positive correlation between 20:5n-3 and 22:6n-3 and total lipid content is also due to physiological processes, as krill are known to conserve these important fatty acids (Cripps & Hill 1998; Stübing & Hagen 2003; Stübing *et al.* 2003; Ju & Harvey 2004; Schmidt *et al.* 2006), and may even synthesize them through elongation and desaturation of 18:3n-3 (Virtue *et al.* 1993a). Because 20:5n-3 and 22:6n-3 are conserved and may not be readily metabolized when the diet changes, they are less useful as phytoplankton biomarkers than fatty acids with weaker correlations to total lipid. Differences in 20:5n-3 and 22:6n-3 levels between krill in different regions, however, indicate that these fatty acids can be used as dietary biomarkers, as long as their physiological role is considered when interpreting data (Clarke 1980). Conversely, levels of 18:4n-3 and 16:1n-7c in krill are reduced when other dietary sources are consumed and this makes them more suitable dietary biomarkers (Stübing *et al.* 2003). This may also explain why quantities of 16:1n-7c and 18:4n-3 were more variable between years and seasons, and why ratios of the diatom/flagellate biomarkers 20:5n-3/22:6n-3 and 16:1n7c/18:4n-3 did not always display the same patterns.

Interannual comparisons between krill caught in the same seasons were carried out between adult krill of similar length, size, and total lipid composition, which is recommended

when drawing conclusions on diet using fatty acid biomarkers (Stübing *et al.* 2003; Schmidt *et al.* 2014).

Despite the overarching seasonal trend of increasing omnivory through to spring seen across the three fishing years, levels of some fatty acids and fatty acid ratios in krill were highly variable between years and fishing locations. For example, diatom biomarkers were very prevalent in winter during some years, as seen in the particularly high ratio of 16:1n-7c/18:4n-3 in South Georgia in 2016, and the degree of herbivory and omnivory within certain locations varied between years. This was particularly evident in 2014 in the WAP samples as krill were predominantly herbivorous, showing significant increases in 22:6n-3 and 18:4n-3 flagellate biomarkers. It should be noted that elevated 22:6n-3 percentages may also be related to reproduction, as a high 22:6n-3/20:5n-3 ratio in gravid females is a predictor of increased hatching success in krill embryos (Yoshida *et al.* 2011). The high 22:6n-3 in krill at the WAP in 2014 suggests some environmental or physiological basis that may not be attributed to diet alone, but the correlation with other herbivory biomarkers suggests it may at least be partially diet related.

Other studies have also investigated interannual differences (Phleger *et al.* 2002; Reiss *et al.* 2015) and regional differences (Schmidt & Atkinson 2016) in krill fatty acid composition. Environmental heterogeneity between different regions in the Southern Ocean has been highlighted as an important driver of krill diet (Reiss *et al.* 2015; Schmidt & Atkinson 2016). South Georgia is considered to be the northern limit of the Antarctic krill habitat, where sea surface temperatures can reach 5°C. It is also ice-free year-round and highly productive, where phytoplankton blooms can last for five months or more and diatoms are often abundant (Schmidt & Atkinson 2016). The WAP region is covered by sea ice in the colder months, phytoplankton blooms may only remain for up to three months (Schmidt *et al.* 2014) and flagellates are often found in the stomachs of krill (Schmidt & Atkinson 2016).

The sampling regime for our study was constrained by fishing operations, therefore it was not possible to obtain samples across all season and location combinations. The implications of this are that seasonal and interannual comparisons may be confounded by differences in fishing locations. Because our overall seasonal patterns of herbivory and omnivory agree with seasonal data from previous studies (which are taken from a range of locations and years), it is likely that season is the major factor driving the fatty acid composition of krill. Abundances of krill prey (phytoplankton and zooplankton) in the Scotia Sea are closely linked to seasonal changes in the extent of sea ice and the light regimes (Murphy *et al.* 2007), which will cause seasonal fluctuations in dietary fatty acid biomarkers in krill. It is evident that varying environmental conditions between different locations and years also influence the proportions of specific fatty acids in krill within the overlying cycle of herbivory-omnivory that appears to be seasonally driven.

One specific biomarker that did not have a strong correlation to any particular season or location was the flagellate biomarker 18:4n-3 that was present at 0.5 – 9.7% of total fatty acids. It was evident from principal component analyses that 18:4n-3 was a major driver of variability within the krill fatty acid profiles. High variability in percentages of 18:4n-3 have previously been reported in juvenile and adult krill collected in summer (0.7 – 11%, Cripps *et al.* 1999; 3.7 – 9.4%, Phleger *et al.* 2002; 0.6 – 8.6%, Schmidt *et al.* 2006), spring and autumn (1.5 – 9%, Hagen *et al.* 2001), and over 11 years in summer and winter (1.0 – 9.2%, Reiss *et al.* 2015). Other studies have found low and less variable percentages of 18:4n-3 in krill (0 – 0.1%, Phleger *et al.* 1998; 1.4 – 2.9%, Atkinson *et al.* 2002; 1.4 – 1.7%, Ju & Harvey 2004; 1 – 2.5%, Schmidt *et al.* 2014). Because the percentages of 18:4n-3 in krill studies to date have ranged between 0 – 11%, it could have a large role in determining the overall fatty acid profile and can be considered a major fatty acid when percentages are high.

The ratio of 16:1n-7c/18:4n-3 in krill was always above 1.0, suggesting that krill preferentially feed on diatoms, but the proportion of flagellate markers to diatom markers increased considerably at numerous times during our three-year study. It may be advantageous for krill to feed on flagellates when they are readily available as they are often abundant at krill study sites (Kopczynska 1992; Cripps & Hill 1998; Schmidt *et al.* 2006). We cannot establish whether an increase in flagellate abundance caused increased feeding, or whether krill may have been targeting flagellates even when they were not abundant.

Flagellates may support krill growth in a manner similar to diatoms (Schmidt *et al.* 2006) even though diatoms are thought to be a superior food source for krill (Pond *et al.* 2005). They may also become increasingly important as a food source for krill in the future, as flagellate abundance is expected to increase with predicted oceanic climate change in some regions of the Southern Ocean (Deppeler & Davidson 2017). Abundances of Southern Ocean flagellates and how they relate to the variability in 18:4n-3 levels in krill from our studied regions needs further investigation.

Our use of high-frequency sampling of catches from the krill fishery has confirmed the overall patterns of seasonal variation of krill fatty acids and diet seen in earlier work. Our approach detected some noteworthy anomalies that would not have been detected if we had only collected samples from one fishing year, location or season. The large spatial and temporal scope of this study highlights the importance of considering interannual and regional variation when investigating differences in the fatty acid content and composition of krill. The study also illustrates the benefits of using fishing vessels to collect scientific samples.

2.6. Acknowledgments

We thank Peter Mansour and Mina Brock for technical assistance in the laboratory and the crew on FV *Saga Sea* for collecting krill samples. This research was funded by an Australian Research Council Linkage Grant LP140100412 between the AAD, CSIRO, IMAS and Aker Biomarine. We thank the two anonymous reviewers and the editors for their helpful comments on the manuscript.

3 New insights into the seasonal diet of Antarctic krill using triacylglycerol and phospholipid fatty acids, and sterol composition

This chapter is under review for *Polar Biology*:

Ericson JA, Hellessey N, Nichols PD, Nicol S, Kawaguchi S, Hoem N, Virtue P (under review). New insights into the seasonal diet of Antarctic krill using triacylglycerol and phospholipid fatty acids, and sterol composition.

3.1. Abstract

Fatty acid analysis is a powerful tool in food web research for estimating dietary sources in marine predators. However, the utility of fatty acids as dietary indicators from whole lipid samples, rather than from separate lipid classes, has been questioned. Samples are often collected at a single time point, precluding seasonal dietary comparisons. We investigated variations in the fatty acid composition of structural (phospholipids) and storage lipids (triacylglycerols) of Antarctic krill (*Euphausia superba*) using fisheries samples obtained over one year. Seasonal variation was observed in fatty acid biomarkers within triacylglycerol and phospholipid fractions of krill. Fatty acids in krill triacylglycerols (thought to better represent recent diet), reflected omnivorous feeding with highest percentages of flagellate biomarkers (18:4n-3) in summer, and diatom biomarkers (16:1n-7c) in autumn, winter and spring. Carnivory biomarkers ($\sum$ 20:1 + 22:1 and 18:1n-9c/18:1n-7c) in krill were greater in autumn. Phospholipid fatty acids were less variable and higher in 20:5n-3 and 22:6n-3, which are essential components of cell membranes. Sterol composition did not yield detailed dietary information, but percentages of the major krill sterol, cholesterol, were significantly higher in winter and spring compared with summer and autumn. Unexpectedly, 18:4n-3 and copepod markers $\sum$ 20:1 + 22:1 were not strongly associated with the triacylglycerol fraction during some seasons. Krill may mobilise 18:4n-3 to phospholipids for conversion to long chain polyunsaturated fatty acids, which would have implications for its role as a dietary biomarker. For the first time, we demonstrate the dynamic seasonal relationship between specific biomarkers and krill lipid classes.

3.2. Introduction

Euphausia superba (Antarctic krill, hereafter 'krill') play a vital role in the Southern Ocean food web, as a lipid-rich food source for higher predators (Murphy *et al.* 1988; Kattner *et al.* 2007). Recent estimates suggest that krill predators consume 55 million tonnes of krill per year in the Scotia Sea region alone (Trathan & Hill 2016). Krill are also targeted by a commercial fishery to produce aquaculture feeds and nutraceutical 'krill oil' products for human consumption, as their lipids (oils) have high levels of essential omega 3 fatty acids 20:5n-3 (eicosapentaenoic acid) and 22:6n-3 (docosahexaenoic acid) (Gigliotti *et al.* 2011; Nicol *et al.* 2012).

Lipids can exceed 40% of the dry mass of krill and the proportion of lipids in krill varies seasonally (Hagen *et al.* 2001). The major lipid classes in krill are triacylglycerols (neutral lipids) and phospholipids (polar lipids), but lower percentages of sterols, monoacylglycerols, diacylglycerols, free fatty acids, sterol esters and wax esters can also be present in the lipid profile (Clarke 1980; Hellessey *et al.* 2018). Triacylglycerol (TAG) percentages increase when food is readily available and krill also utilise TAG for energy (Mayzaud *et al.* 1998), therefore, percentages decrease at times when food is scarce or when excess energy is needed during the reproductive period (Falk-Petersen *et al.* 2000). Phospholipids (PL) (such as phosphatidyl-ethanolamine; see Mayzaud 1997) are tightly conserved by krill (Virtue *et al.* 1993a) as they have an important structural role in cell membranes and control membrane fluidity (Parrish *et al.* 2000). The phosphatidyl-choline fraction of the phospholipids may also be used as storage lipid (Mayzaud 1997; Hagen *et al.* 2001). Reasons for the extra storage capacity of phosphatidyl-choline are unknown, but may be linked to reproductive processes and development (Mayzaud 1997).

Percentages of TAG determine the overall lipid class composition (Fricke *et al.* 1984; Hellessey *et al.* 2018), because TAG is the primary depot lipid in krill (Clarke 1984; Falk-

Petersen *et al.* 2000; Schmidt & Atkinson 2016). Triacylglycerol percentages increase between summer and late autumn when krill are actively feeding, and decrease throughout winter and early spring. Phospholipids fluctuate inversely to TAG, and krill generally have highest percentages of PL in winter and early spring (Hellessey *et al.* 2018).

Krill lipids are composed of polyunsaturated (PUFA), monounsaturated (MUFA) and saturated (SFA) fatty acids, and their fatty acid content and composition has been well studied (Bottino 1974; Fricke *et al.* 1984; Falk-Petersen *et al.* 2000; Hagen *et al.* 2001; O'Brien *et al.* 2011; Schmidt & Atkinson 2016; Ericson *et al.* 2018a). Selected fatty acids can be used as dietary 'biomarkers' as they are primarily derived from the diet of krill (Stübing & Hagen 2003; Schmidt & Atkinson 2016). The fatty acids 20:5n-3 and 16:1n-7c have been used as biomarkers for diatoms, while 22:6n-3 and 18:4n-3 have been used as flagellate biomarkers (Stübing & Hagen 2003; Ericson *et al.* 2018a). The ∑ 20:1 + 22:1 isomers are elevated in some Antarctic copepods (e.g. *Metridia gerlachei* (Ju & Harvey 2004) and *Calanoides acutus* (Hagen *et al.* 1993; Graeve *et al.* 1994; Ju & Harvey 2004)) and may be used as indicators of copepod consumption by krill (Kattner & Hagen 1995; Ju & Harvey 2004). Higher ratios of 18:1n-9c / 18:1n-7c also infer greater carnivory (Stübing & Hagen 2003).

The fatty acid profile of the total lipid can be used to investigate the diet of krill and this is preferred in studies with large sample sizes, as the separation of lipid classes is laborious (Stübing & Hagen 2003). In studies with smaller sample sizes, the krill lipid classes can be separated and the fatty acid profile is obtained for each lipid class. Fatty acid biomarkers located in the TAG lipid fraction are thought to be the best indicators of krill diet (Bottino 1974; Virtue *et al.* 1993a). Studies most often use fatty acid data from the total lipid of whole krill or dissected body parts (Virtue *et al.* 1993b, 1996; Atkinson *et al.* 2002; Alonzo *et al.* 2003, 2005a; Schmidt *et al.* 2006; O'Brien *et al.* 2011; Schmidt *et al.* 2014; Auerswald *et al.* 2015; Virtue *et al.* 2016; Ericson *et al.* 2018a) or fatty acid data from the TAG fraction only

(Ju & Harvey 2004; Reiss *et al*. 2015) to make inferences about the diet of krill. A lesser number of early studies also reported the fatty acid composition of the more tightly conserved PL fraction (Clarke 1980; Fricke *et al*. 1984; Virtue *et al*. 1993a; Falk-Petersen *et al*. 2000; Mayzaud *et al*. 2000; Hagen *et al*. 2001; Stübing *et al*. 2003).

Sterol composition can also be used to make inferences about an organism's diet, and can complement fatty acid biomarker data (Virtue *et al*. 1993a, 1993b; Martin-Creuzburg & von Elert 2009). Cholesterol is the major sterol in crustaceans, comprising up to 95% of the sterol profile (Kanazawa 2001). Minor sterols of algal origin are often used as dietary biomarkers (Parrish *et al*. 2000).

A recent study (Ericson *et al*. 2018a) provides data on the seasonal and interannual fluctuations in fatty acid composition of the total lipid of krill. The present study further investigates the fatty acid composition of the separate lipid classes (TAG and PL) in krill, to establish whether lipid class separation is necessary when using fatty acids as dietary biomarkers. There is currently limited data on how the distribution of krill fatty acid biomarkers in the TAG and PL of krill varies between seasons, and no collated data on seasonal fluctuations in krill sterols. Seasonal studies have been difficult because of a lack of samples from seasons other than summer, and because at-sea scientific sampling rarely covers the entire seasonal cycle in sufficient detail.

We utilised samples collected by a krill fishing vessel during 2016 to obtain comprehensive seasonal data on the sterol composition of krill, and the distribution of fatty acids in the major lipid classes.

3.3. Materials and Methods

3.3.1. Krill sample collection

Krill sample collection is described in detail in Ericson *et al.* (2018a). Briefly, krill were caught on board the FV *Saga Sea* (Aker Biomarine) during their 2016 fishing season (December 2015 – September 2016), from three different locations; the West Antarctic Peninsula (WAP), South Orkney Islands (SOI) and South Georgia (SG) (Figure 3.1). Twenty krill day $^{-1}$ were randomly sampled from the catch by a fisheries observer (there was no selection by size or maturity stage). These samples were transported to Hobart, Tasmania on dry ice and stored at –80ºC.

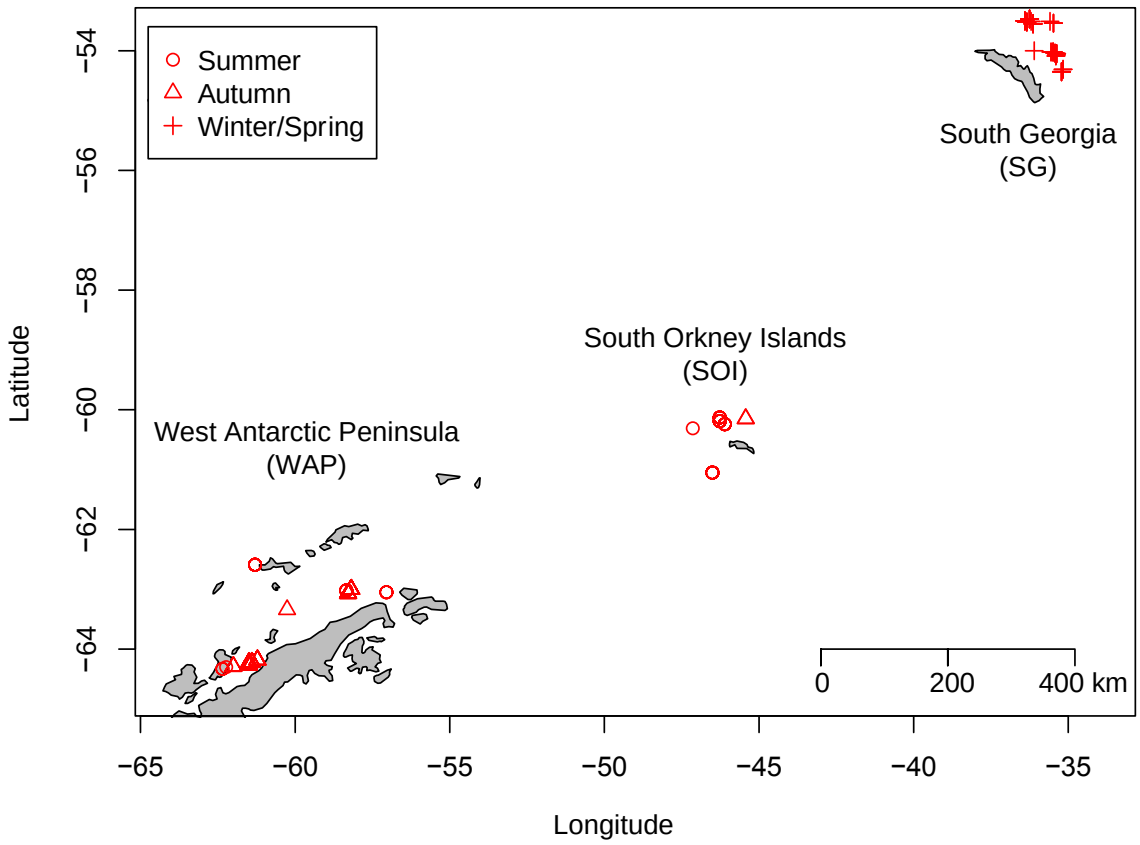


Figure 3.1. Map of locations where krill samples were collected by the krill fishery in (○) summer (Jan – Feb), (△) autumn (Mar – May), and (+) winter/spring (Jun – Sep) of 2016, at the West Antarctic Peninsula (WAP), South Orkney Islands (SOI) & South Georgia (SG). Map modified from Hellessey *et al.* (2018).

3.3.2. Initial lipid extraction and fatty acid analyses

Three male and three female krill were collected from the fisheries samples at 2-week intervals, for lipid extraction. Krill were individually extracted in separation funnels using a modified method of Bligh and Dyer (1959), with a solvent mixture of methanol (MeOH)/dichloromethane (CH_2Cl_2)/water (H_2O) at 20:10:7, by vol. To separate phases, 10 mL CH_2Cl_2 and 10 mL saline MilliQ H_2O were added the following day. The lower lipid layer was drained into a round bottomed flask and solvent was removed using a rotary evaporator, to concentrate the total lipid extract (TLE). The TLE was stored at –20 ºC in a pre-weighed glass vial with added solvent (CH_2Cl_2), to ensure that oxidation of the sample did not occur.

These total lipid extracts (TLE) obtained from Ericson *et al*. (2018a) were used for the present study. Krill with a large range of selected biomarker percentages in the TLE were included. Only males were included to eliminate gender as a potential confounding variable (Clarke 1980; Mayzaud *et al*. 2000). Males that had < 25% TAG (as % of total lipids) were excluded, as low TAG percentages during the reproductive season may make them less suitable for dietary analysis (Virtue *et al*. 1996; Stübing & Hagen 2003). Total lipid extracts from 12 males from the 2016 catch were selected from summer, autumn and combined winter/early spring (total $N = 36$). Summer krill were sampled from the WAP and the SOI in January and February 2016, autumn krill were sampled from the WAP between March - May 2016, and winter/early spring (referred to as 'winter/spring' hereafter) krill were sampled from SG between June – September 2016. Samples selected for this study are shown in Appendix IV, along with their total lipid (mg g^{-1} DM; dry mass), TAG and PL percentage data (% of total lipid) obtained from their TLE. Detailed analyses for the full suite of krill collected in 2016 can be found in Hellessey *et al*. (2018).

3.3.3. Separation of lipid classes via column chromatography

Aliquots were taken from the TLE and analysed via column chromatography, to investigate the fatty acid composition within the lipid classes of krill. For all chosen samples (N = 36), one gram of activated silica was added to a glass column and washed through using chloroform ($CHCl_3$) to pack the column. Ten milligrams of total lipid extract were added to the packed column. Triacylglycerols were eluted with 10 ml $CHCl_3$, followed by elution of glycolipids with 20 ml acetone (C_3H_6O), and elution of phospholipids with 20 ml methanol (MeOH), to produce extracts for triacylglycerol, phospholipid and glycolipid fractions (total lipid class fractions; TLCF). All TLCF were reduced via rotary evaporation and added to 1.5 ml glass vials with Teflon caps. Accurate lipid class separation was confirmed by running 1 µl aliquots of all lipid class fractions through an Iatroscan TLC-FID analyser (see Hellessey *et al*. 2018 for detailed methods) following column chromatography. Once accurate separation was verified, TAG and PL lipid fractions were used to prepare fatty acid methyl esters (FAME) for fatty acid analysis.

A subsample of each TLCF was transferred to a glass test tube with a Teflon® screw-cap and 3 mL of methylating solution (MeOH /CH_2Cl_2/hydrochloric acid (HCl), 10:1:1, by vol) was added. Each test tube was then heated at 90 – 100 ºC for 75 min, then cooled for 5 min before addition of 1mL H_2O and 1.8 mL hexane (C_6H_{14})/CH_2Cl_2 solution (4:1, by vol) to extract the FAME. Samples were centrifuged for 5 min, and the upper layer (FAME) was transferred to a vial. An additional 1.8 mL of C_6H_{14}/CH_2Cl_2 solution was added to the test tube, and the sample was centrifuged again, before adding the top layer of FAME to the vial. This process was carried out three times in total, to ensure that all of the FAME had been extracted and added to the vial (samples in the vial were blown down with nitrogen (N_2) gas in between transfers).

To prepare samples for gas chromatography (GC-FID), 1.5 mL of internal injection standard (23:0 FAME) was added to each vial. Samples were analysed via GC-FID using an Agilent Technologies 7890A System equipped with a non-polar Equity®-1 fused silica capillary column (15 m length x 0.1 mm internal diameter x 0.1 μm film thickness). Samples (0.2 μl) were injected in spitless mode at an oven temperature of 120 ºC with helium the carrier gas. The oven temperature was raised at a rate of 10 ºC min^{-1} up to 270 ºC, then a rate of 5 ºC min^{-1} up to 310 ºC. Quantification of fatty acid peaks (expressed as a % of the total fatty acid area) was conducted using Agilent Technologies ChemStation software. Initial identification was based on comparison of retention times with known (Nu Check Prep) and fully characterized laboratory (tuna oil) standards.

Gas chromatography-mass spectrometry (GC-MS) was carried out using a Thermo Scientific 1310 GC-MS coupled with a TSQ triple quadruple, to further confirm component identification. Selected samples were injected using a Tripleplus RSH auto sampler using a non-polar HP-5 Ultra 2 bonded-phase column (50 m length x 0.32 mm internal diameter x 0.17 μm film thickness). The HP-5 column was a similar polarity to the column used for GC-FID analyses. The initial oven temperature (45 ºC) was held for 1 min, then rose at a rate of 30 ºC min^{-1} to 140 ºC, then at a rate of 3 ºC min^{-1} to 310 ºC, and held for 12 min. Helium was the carrier gas. Operating conditions of the GC-MS were as follows: electron impact energy 70 eV; emission current 250 μamp; transfer line 310 ºC; source temperature 240 ºC; scan rate 0.8 scans sec^{-1}; mass range 40 – 650 Da. Mass spectra were acquired and processed with the software Thermo Scientific Xcalibur™. Nu Check Prep and tuna oil standards were also used for assistance in identification of peaks.

3.3.4. Sterol analysis

An additional 300 µl aliquot was taken from each of the TAG fractions for saponification. Each aliquot was transferred into a glass test tube fitted with a Teflon lined screw cap, blown down under N_2 gas and treated with 2 mL of saponifying solution (5% potassium hydroxide (KOH) in MeOH/MilliQ H_2O, 80:20, by vol), then heated at 60 °C for 3 h. Samples were cooled and 1 ml of MilliQ H_2O and 1.8 ml of C_6H_{14} : CH_2Cl_2 solution was added to extract the total non-saponifiable neutral lipids (TSN). Samples were then centrifuged for 5 min and the upper layer containing TSN was transferred to a vial, and another 1.8 ml of C_6H_{14}/CH_2Cl_2 was added to the test tube. This process was carried out three times and samples were blown down each time using N_2 gas.

Samples of TSN lipids obtained above were silylated by treatment with N_2 gas and addition of 50 µl N,O-bis (Trimethylsilyl) trifluoroacetamide, then heated overnight at 60 °C. Prior to analysis, samples were blown down using N_2 gas and 1000 µl of internal injection standard (23:0 FAME) was added to each vial. Samples were blown down again under N_2 gas and transferred to glass inserts with 200 µl CH_2Cl_2. Samples were then run through a GC-FID and GC-MS as described above, to obtain sterol composition and content, and to confirm component identifications.

3.3.5. Statistical analyses

Principal components analysis (PCA) of fatty acid data was carried out in PRIMER 6 using Pearson correlation, due to large differences in fatty acid variances. Data was transformed (log x+1) prior to PCA analysis. Fatty acid biomarker data was analysed in RStudio (version 1.1.453) using one-way ANOVA with either season or lipid class as a factor, or two-way ANOVA with season and lipid class as factors, and a season*lipid class interaction. Tukey comparisons were used to investigate significant differences between levels of season. Sterol

data was also analysed in RStudio, using one way ANOVA with season as a factor. Data for all analyses was log or square root transformed when it did not meet assumptions of normality or homogeneity of variances. A Welch's test was used for sterol data that had heterogeneous variances and data transformation did not normalise the data.

3.4. Results

The fatty acid composition (as a percentage of total fatty acids) of the TAG and PL fractions of krill in summer, autumn and winter/spring of 2016 are shown in Table 3.1. Total percentages of omega-3 fatty acids, polyunsaturated (PUFA), monounsaturated (MUFA) and saturated (SFA) fatty acids are also shown in Table 3.1.

Principal components analysis (PCA) of krill from all seasons combined is shown in Figure 3.2. The fatty acid composition (%) of TAG and PL in krill are clearly separated along PC1, which explains 85.3% of the variation. Phospholipids were higher in the long chain omega-3 PUFA, particularly 20:5n-3 and 22:6n-3. Triacylglycerols were higher in the SFA 14:0, and MUFA 16:1n-7c and 18:1n-9c. PC2 explains a minor (5.9%) amount of the fatty acid variation, separating krill with higher percentages of 18:4n-3 from krill with higher percentages of 20:1n-9c and 22:1n-9c. The fatty acid 16:0 was evenly distributed between TAG and PL with substantial percentages (~20%) in both lipid classes.

Table 3.1. Fatty acid composition (%) of the triacylglycerols (TAG) & phospholipids (PL) of *Euphausia superba* (mean ± SD) in summer, autumn & winter/spring of 2016. Only fatty acids > 0.5% are shown & fatty acids < 0.5% are classed as "other". For each lipid class & season $n = 4$.

Fatty acid	Summer		Autumn		Winter/Spring	
	TAG	PL	TAG	PL	TAG	PL
14:0	11.3 ± 1.7	1.3 ± 0.3	9.8 ± 3.8	0.8 ± 0.4	12.8 ± 2.1	1.2 ± 0.4
16:4	1.5 ± 0.7	0.1 ± 0.1	1.5 ± 0.6	0.1 ± 0.04	0.9 ± 0.4	0.03 ± 0.01
16:1n-7c	10.4 ± 1.6	1.5 ± 0.3	12.6 ± 1.3	1.6 ± 0.3	12.7 ± 1.1	1.6 ± 0.3
16:0	21.0 ± 1.9	21.1 ± 1.5	21.9 ± 1.8	20.5 ± 1.9	20.5 ± 4.6	22.8 ± 5.1
18:4n-3	5.0 ± 2.5	3.2 ± 1.2	2.4 ± 0.9	2.3 ± 0.6	1.8 ± 1.4	1.3 ± 0.4
18:2n-6	1.3 ± 0.5	1.1 ± 0.3	0.9 ± 0.4	0.9 ± 0.5	1.7 ± 0.6	1.9 ± 0.4
18:3n-3	0.9 ± 0.9	1.1 ± 0.8	0.4 ± 0.2	0.7 ± 0.3	0.5 ± 0.4	1.0 ± 0.2
18:1n-9c	20.2 ± 3.4	4.7 ± 1.1	25.2 ± 4.7	4.7 ± 0.6	22.0 ± 4.5	4.9 ± 1.2
18:1n-7c	6.9 ± 1.5	5.3 ± 0.5	7.4 ± 0.6	5.6 ± 0.5	9.0 ± 2.0	5.1 ± 0.5
18:0	2.2 ± 0.3	1.6 ± 0.3	2.0 ± 0.3	1.4 ± 0.2	2.1 ± 0.4	1.2 ± 0.2
20:5n-3	8.4 ± 2.7	33.0 ± 4.5	6.7 ± 2.8	36.6 ± 4.0	5.2 ± 1.8	31.7 ± 2.9
20:1n-9c	1.4 ± 0.4	0.8 ± 0.3	1.9 ± 0.3	1.2 ± 0.2	1.6 ± 0.5	0.8 ± 0.3
20:1n-7c	1.4 ± 0.4	0.3 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	0.4 ± 0.1
21:5n-3	0.4 ± 0.2	1.4 ± 0.2	0.3 ± 0.1	1.7 ± 0.5	0.2 ± 0.1	0.9 ± 0.1
22:6n-3	3.1 ± 1.1	16.6 ± 3.7	1.7 ± 0.5	14.3 ± 2.3	2.5 ± 1.1	16.0 ± 2.3
22:5n-3	0.2 ± 0.1	0.8 ± 0.1	0.2 ± 0.1	1.7 ± 0.5	0.2 ± 0.1	1.0 ± 0.1
22:1n-9c	0.5 ± 0.2	1.5 ± 0.7	0.7 ± 0.2	2.7 ± 0.4	0.5 ± 0.2	2.0 ± 0.7
Other *	5.1 ± 1.5	4.9 ± 1.1	4.0 ± 0.7	3.9 ± 0.9	5.3 ± 1.2	6.2 ± 1.0
Sum n-3	18.3 ± 5.4	56.5 ± 3.7	11.8 ± 4.0	56.8 ± 3.0	10.6 ± 4.6	52.4 ± 4.8
Sum PUFA	22.5 ± 6.4	59.8 ± 3.4	15.3 ± 4.7	59.2 ± 3.0	14.2 ± 5.8	56.1 ± 4.9
Sum MUFA	41.9 ± 4.8	16.0 ± 1.9	50.2 ± 4.1	18.0 ± 1.1	48.9 ± 3.5	17.6 ± 2.3
Sum SFA	35.6 ± 2.6	24.7 ± 2.0	34.6 ± 4.9	23.3 ± 2.2	37.0 ± 4.0	26.7 ± 5.0

* Other fatty acids: i14:0, 14:1, i15:0, a15:0, 15:0, 16:3, i16:0, 16:1n-9c, 16:1n-7t, 16:1n-5c, 16:1n-13t, 16:0FALD, i17:0, 17:1n-8c + a17:0, 17:1, 17:0, 18:3n-6, i18:0, 18:1n-7t, 18:1n-5c, 18:1, 19:1, 19:0, 20:4n-6, 20:3n-6, 20:4n-3, C20PUFA, 20:2n-6, 20:1n-11c, 20:1n-5c, 20:0, 21:0, 22:5n-6, 22:4n-6, 22:1n-11c, 22:1n-7c, 22:0, 24:1n-11c, 24:1n-9c, 24:1n-7c, 24:0, phytanic acid.

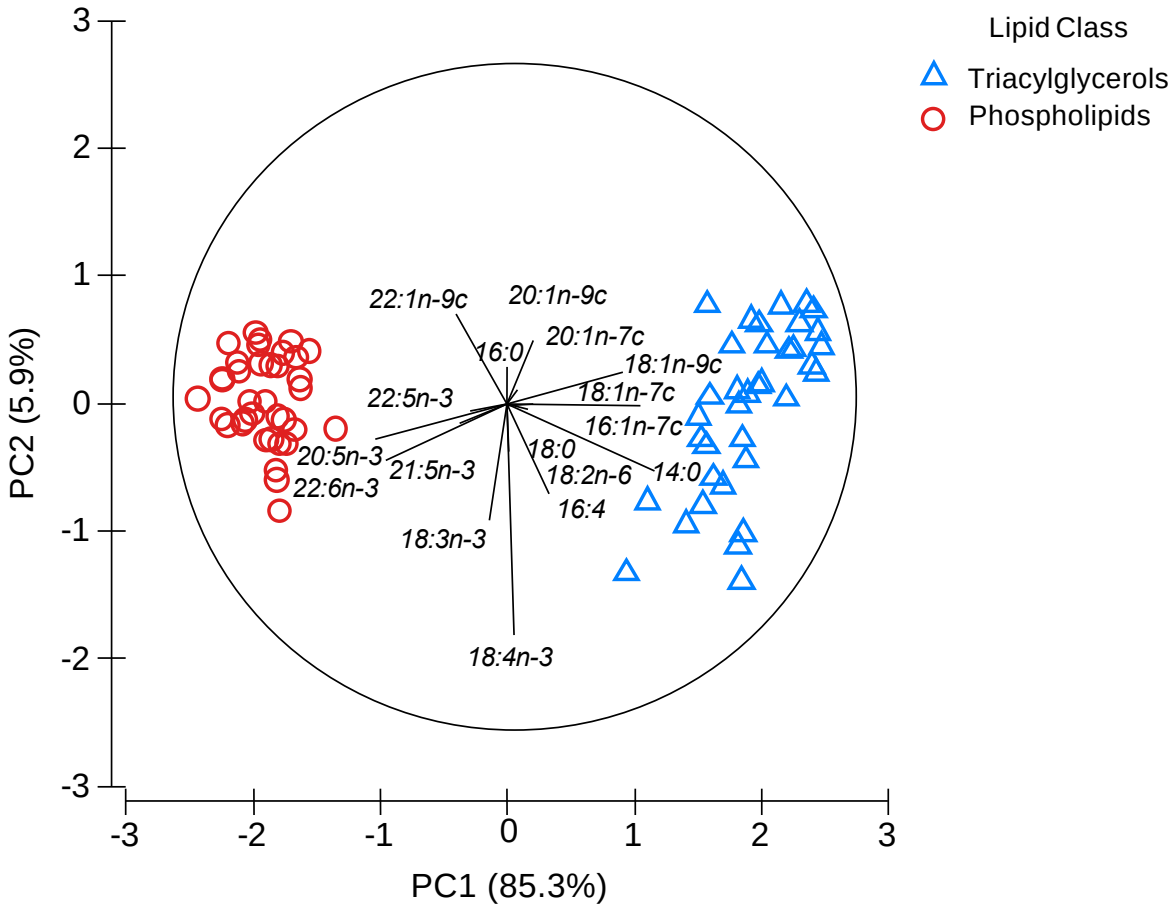


Figure 3.2. Principal components analysis (PCA) of the fatty acid composition (%) of triacylglycerols (TAG) & phospholipids (PL) of male *Euphausia superba*. Only fatty acids >0.5% were used for PCA. Data for krill from all seasons are combined.

3.4.1. Composition of fatty acid biomarkers in the triacylglycerols and phospholipids of krill

Percentages (mean ± SE) of the major fatty acid biomarkers in the TAG and PL of krill are shown in Figure 3.3. Percentages of essential omega 3 PUFA 20:5n-3 and 22:6n-3 and carnivory markers ∑ 20:1 + 22:1 in krill were higher in PL, while percentages of phytoplankton biomarkers 16:1n-7c, 18:4n3 and the carnivory ratio 18:1n9-c / 18:1n-7c were higher in TAG (Figure 3.3). Percentages of 18:4n-3 in both lipid classes were more variable than the other fatty acid biomarkers (Figure 3.3).

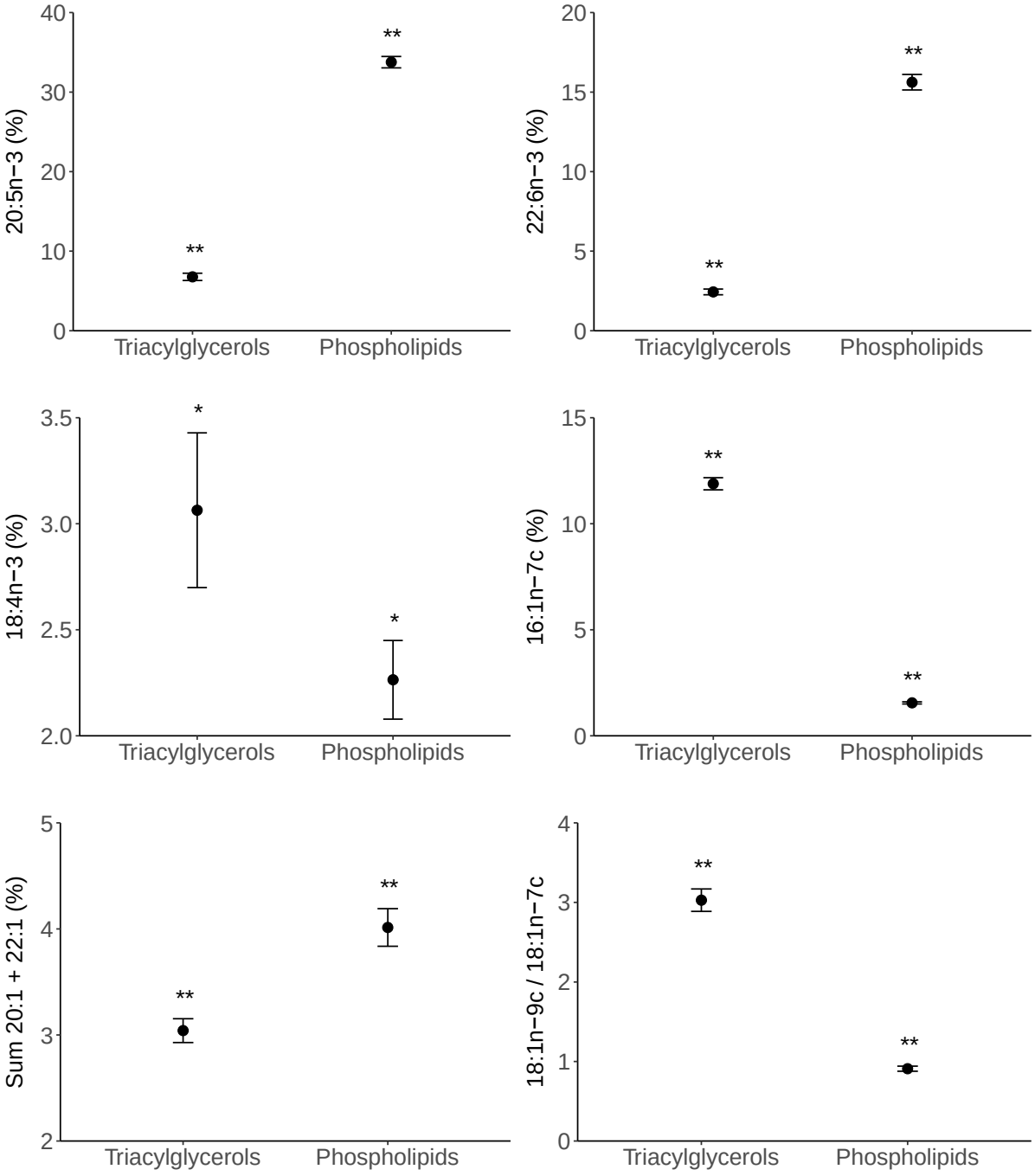


Figure 3.3. Percentages (mean ± SE) of fatty acid biomarkers 20:5n-3, 22:6n-3, 18:4n-3, 16:1n-7c, ∑ 20:1 + 22:1, & ratios of 18:1n-9c/18:1n-7c in male *Euphausia superba*, in triacylglycerols (TAG) & phospholipids (PL). Data from all seasons are combined. Significant differences between the TAG and PL are denoted by asterisks where * = $p < 0.05$, ** = $p < 0.001$. For each lipid class $n = 3$.

The composition of fatty acid biomarkers in TAG and PL also varied seasonally (Figure 3.4). Percentages of 20:5n-3 in krill were higher in TAG in summer than in winter/spring (One Way ANOVA, df = 2, Tukey $p = 0.007$) and higher in PL in the autumn than in winter/spring (One Way ANOVA, df = 2, Tukey $p = 0.011$). Percentages of 22:6n-3 were lower in TAG in autumn compared with the other seasons (One Way ANOVA, df = 2, Tukey $p < 0.04$), while there were no seasonal differences in the PL (One Way ANOVA, df = 2, $p = 0.134$).

Percentages of 18:4n-3 within both TAG and PL were higher in summer than in winter/spring (One Way ANOVA, df = 2, Tukey $p < 0.001$), with intermediate percentages in autumn (Figure 3.4). The TAG and PL of krill had similar percentages of 18:4n-3 in autumn and winter/spring (Two Way ANOVA Lipid Class*Season, df = 2, Tukey $p > 0.925$). Percentages of 16:1n-7c in TAG were lowest in summer (One Way ANOVA, df = 2, Tukey $p < 0.001$), while no seasonal differences were observed in PL (One Way ANOVA, df = 2, Tukey $p = 0.368$).

Seasonal differences were also observed for the carnivory biomarkers $\sum$ 20:1 + 20:1 and 18:1n-9c/18:1n-7c. The $\sum$ 20:1 + 22:1 in TAG were lower in summer than the other seasons (One Way ANOVA, df = 2, Tukey $p < 0.04$) and highest in PL in autumn (One Way ANOVA, df = 2, Tukey $p < 0.02$). Ratios of 18:1n-9c/18:1n-7c in TAG were higher in autumn than in winter/spring (One Way ANOVA, df = 2, Tukey $p = 0.042$), but no seasonal differences were seen in PL (One Way ANOVA, df = 2, Tukey $p = 0.311$).

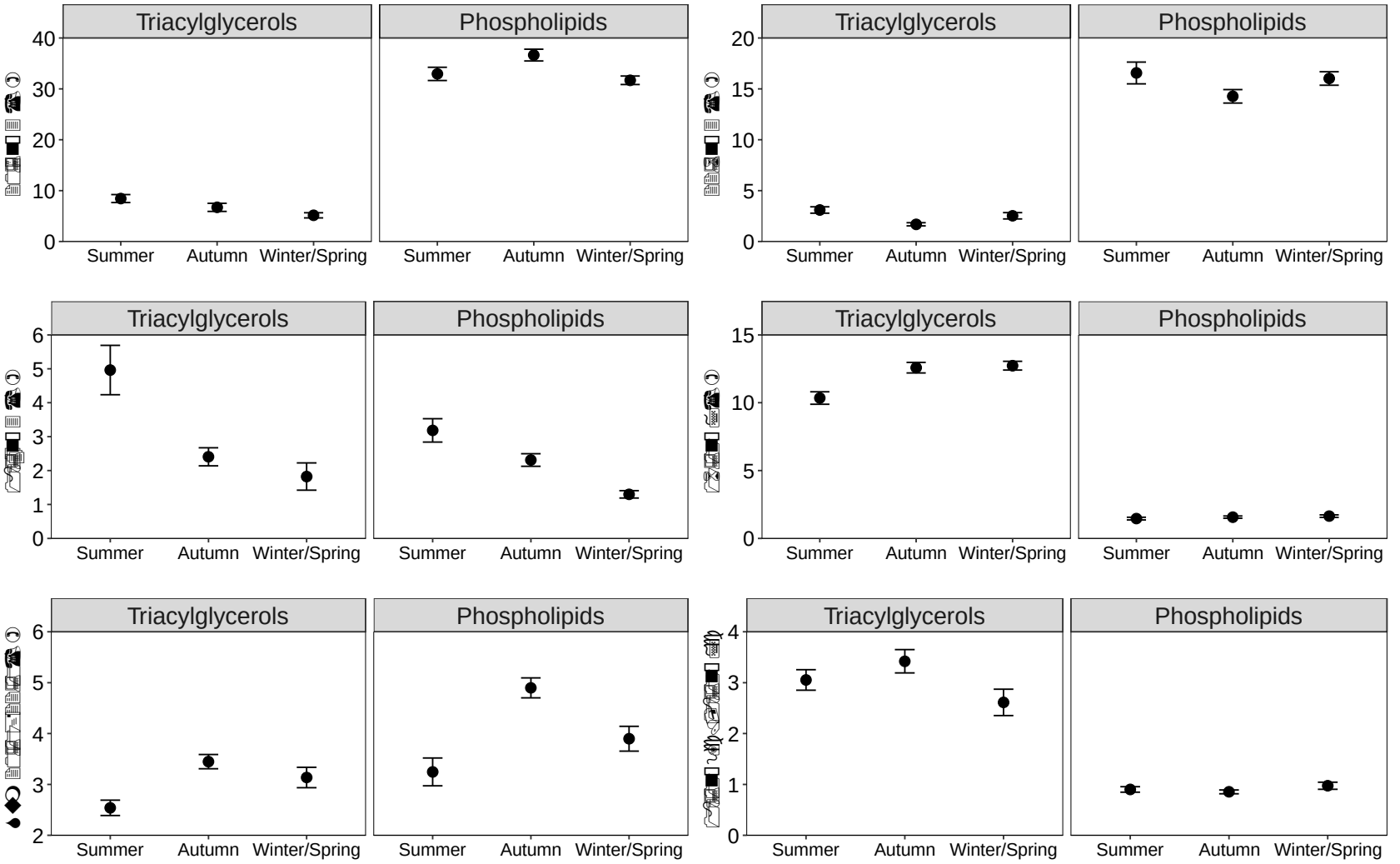


Figure 3.4. Percentages (mean ± SE) of fatty acid biomarkers 20:5n-3, 22:6n-3, 18:4n-3, 16:1n-7c, ∑ 20:1 + 22:1, & ratios of 18:1n-9c/18:1n-7c in triacylglycerols (TAG) & phospholipids (PL) of male *Euphausia superba*, in summer (Jan – Feb), autumn (Mar – May) & winter/spring (Jun – Sep) of 2016. For each lipid class & season *n* =12.

The relationship between TAG and PL fatty acid biomarkers in krill is summarised by the TAG/PL ratios in Table 3.2. The high ratios of 16:1n-7c and 18:1n-9c / 18:1n-7c in TAG, and strong association of 20:5n-3 and 22:6n-3 with PL, have also been consistently observed in other studies (see references in Table 3.2). The ratios of 18:4n-3 and ∑ 20:1 + 20:1 isomers in TAG and PL are variable and these biomarkers do not show a consistent association with either TAG or PL. Percentages of 18:4n-3 are consistently higher in TAG of krill in summer, but this relationship does not hold in autumn or in studies when the season is not stated (Table 3.2). The ratio of ∑ 20:1 + 22:1 isomers in TAG/PL is highly variable between studies, ranging from 0.7 – 7.7 (Table 3.2).

3.4.2. Seasonal variations in krill sterols

Four sterols were identified in the neutral lipids of krill (Table 3.3). Cholesterol (cholest-5-en-3β-ol) was the major sterol, making up 50 – 89% of the sterol composition, while desmosterol (cholesta-5,24-dien-3β-ol) made up 11 – 33% of the composition. Brassicasterol (24-methylcholesta-5,22E-dien-3β-ol) percentages were variable, and present at 0 – 12% of sterols. Thirty-three of the sampled krill had brassicasterol percentages < 5%, but three krill sampled in autumn had percentages exceeding 9%. Trans-22-dehydrocholesterol (24-cholesta-5,22E-dien-3β-ol) was a minor component making up 0 – 3% of the total sterol composition. Other unidentified sterols made up 0 – 13% of the sterol composition.

Average cholesterol levels were 9 – 14 % higher in winter/spring compared with summer and autumn, while desmosterol percentages were lowest in winter/spring (Table 3.3). Average percentages of brassicasterol were highest in autumn, but seasonal differences were not statistically significant due to large variances in autumn data. Trans-22-dehydrocholesterol percentages were lowest in autumn (Table 3.3).

Table 3.2. Triacylglycerol/phospholipid (TAG/PL) ratios of selected fatty acid biomarkers (diatom markers 16:1n-7c, 20:5n-3; flagellate markers 18:4n-3, 22:6n-3; carnivory markers 18:1n-9c / 18:1n-7c, ∑ 20:1 + 22:1 isomers) in whole male *Euphausia superba* samples from different studies. Values > 1.0 indicate higher percentages in the TAG; values = 1.0 indicate the same percentages in both lipid classes; values < 1.0 indicate higher percentages in the PL. Overall relationships between the ratios of each biomarker in TAG and PL are summarised in the bottom row of the table where T > P = TAG have consistently higher percentages. T < P = PL have consistently higher percentages. NCR = no consistent relationship. Sample size = '*n*'.

16:1n-7c	**18:4n-3**	**20:5n-3**	**22:6n-3**	**∑ 20:1 + 22:1**	**18:1n-9c / 18:1n-7c**	**Season**	**Sex**	***n***	**Reference**
8.0	4.0	0.1	0.3	-	3.2	Summer	Unknown	Unknown	Falk-Petersen *et al*. 2000
3.8	4.5	0.5	0.1	7.7	1.9	Summer	Males	2	Clarke 1980
6.9	1.6	0.3	0.2	0.8	3.4	Summer	Males	12	Ericson *et al*. (this study)
2.4	-	0.1	<0.08	2.2	1.7	Summer	Unknown	3	Fricke *et al*. 1984*
3.0	1.0	0.1	0.4	-	2.5	Autumn	Unknown	Unknown	Falk-Petersen *et al*. 2000
7.9	1.0	0.2	0.1	0.7	3.8	Autumn	Males	12	Ericson *et al.* (this study)
6.0	-	0.04	0.06	0.7	2.0	Autumn	Unknown	3	Fricke *et al*. 1984*
5.2	0.8	0.1	0.1	-	2.4	Autumn (field)	Females	3	Stübing *et al*. 2003**
5.0	1.2	0.1	0.1	-	2.6	Autumn (field)	Juveniles	3	Stübing *et al*. 2003**
7.9	1.4	0.2	0.2	0.8	2.6	Winter/Spring	Males	12	Ericson *et al*. (this study)
3.9	1.7	0.1	0.2	-	1.1	Lab study	Unknown	3	Virtue *et al*. 1993a
4.9	0.7	0.03	0.1	-	3.7	Unknown	Males	2	Hagen *et al*. 2001
3.4	0.8	0.1	0.1	-	2.8	Unknown	Females	3	Hagen *et al*. 2001
T > P	**NCR**	T < P	T < P	**NCR**	T > P				

* = Fatty acids (%) for phospholipids are from phosphatidyl-choline fraction only.

** = Fatty acids (%) for phospholipids are from phosphatidyl-choline fraction only. Percentages of TAG and PL were estimated from Figure 4 in Stübing *et al.* (2003) as no table of exact percentages was provided.

- = Could not be calculated from the available data.

Table 3.3. Percentages of sterols (mean ± SD) in male *Euphausia superba* sampled in summer (Jan – Feb), autumn (Mar – May) & winter/spring (Jun – Sep) of 2016. Statistical results for One Way ANOVA Tukey comparisons & Welch tests are shown (df = degrees of freedom). Significant differences are denoted by values that do not share lower case letters.

	Summer	Autumn	Winter/Spring	Statistical test and *p* value
Cholesterol cholest-5-en-3β-ol	73.7 ± 5.5 [a]	68.9 ± 10.1 [a]	82.4 ± 4.4 [b]	df = 2, Tukey $p < 0.015$
Desmosterol cholesta-5,24-dien-3β-ol	22.2 ± 5.2 [a]	23.9 ± 4.1 [a]	14.9 ± 2.0 [b]	df = 2, Tukey $p < 0.001$
Brassicasterol 24-methylcholesta-5,22E-dien-3β-ol	1.0 ± 2.1 [a]	3.1 ± 4.7 [a]	0.7 ± 1.5 [a]	df = 2, Welch test $p = 0.263$
Trans-22- dehydrocholesterol 24-cholesta-5,22E-dien-3β-ol	1.3 ± 0.7 [a]	0.4 ± 0.4 [b]	1.3 ± 0.9 [a]	df = 2, Tukey $p < 0.001$
Other (unidentified sterols)	1.9 ± 2.9 [a]	3.7 ± 4.8 [a]	0.8 ± 1.8 [a]	df = 2, Tukey $p > 0.078$

3.5. Discussion

The composition of fatty acids and fatty acid biomarkers in krill differed between lipid classes (TAG and PL) and seasons (summer, autumn and winter/spring). The strong association of 20:5n-3 and 22:6n-3 with PL is consistent with previous studies (Clarke 1980; Fricke *et al.* 1984; Virtue *et al.* 1996; Mayzaud *et al.* 2000; Hagen *et al.* 2001; Stübing *et al.* 2003; Alonzo *et al.* 2005b), as these long chain PUFA have an important structural role in the cell membranes and are tightly conserved (Dalsgaard *et al.* 2003). Higher percentages of MUFA and SFA in the TAG fraction, namely 14:0, 16:1n-7c and 18:1n-9c, are also consistent with previous studies (Falk-Petersen *et al.* 2000; Hagen *et al.* 2001; Alonzo *et al.* 2005b). More variable and less predictable percentages of 18:4n-3 in krill (compared with other biomarkers) have also been previously reported (Schmidt *et al.* 2006; Ericson *et al.* 2018a).

Seasonal changes in TAG fatty acids provided inferences about the diet of krill. The 1.2 – 1.8-fold increase in 20:5n-3 and 22:6n-3 in TAG of krill in summer indicated that higher percentages of phytoplankton were consumed during summer. The biomarkers 18:4n-3 and 16:1n-7c were more seasonally variable in TAG, with highest percentages of 18:4n-3 in summer and highest percentages of 16:1n-7c in autumn and winter/spring. This suggests that krill were feeding on higher percentages of flagellates in the summer, and switched to a more

diatom enriched diet in the autumn and winter. Elevated summer and autumn ratios of 18:1n-9c / 18:1n-7c in TAG of krill indicated that some carnivorous feeding was occurring during these seasons, while copepods (∑ 20:1 + 22:1 isomers in TAG) comprised a higher proportion of the diet in autumn and winter/spring. It should be noted that high percentages of 18:1n-9c have also been found in krill fed cryptophytes (Alonzo *et al.* 2005a) and krill are able to biosynthesize it (Reiss *et al.* 2015), therefore, this may partially confound its use as a carnivory marker.

Because the sampling regime for this study was dictated by fishing operations, samples could not be collected for all season and location combinations. This implies that although these dietary signatures show seasonal trends, they may also reflect the region in which the krill were caught (Reiss *et al.* 2015). The ocean surrounding South Georgia is known to have high concentrations of diatoms (Schmidt & Atkinson 2016), which may also explain the high percentages of 16:1n-7c in the winter/spring krill caught there. In the WAP region, flagellates are also often found in krill stomachs (Schmidt & Atkinson 2016), and krill caught in this region had elevated percentages of the flagellate marker 18:4n-3. Changes in the fine-scale fatty acid composition of krill are likely to reflect a range of spatial and temporal factors including season and location. However, the overarching trend of increasing omnivory from summer through to winter is likely to be seasonally driven, as abundances of krill prey are closely linked to seasonal changes in their environment (Murphy *et al.* 2007). Our observed pattern of increasing omnivory from summer through to autumn and winter also agrees with previous seasonal studies conducted at a range of locations (see review in Schmidt & Atkinson 2016). On a larger scale, the specific fatty acid composition of krill is also known to differ interannually (Ericson *et al.* 2018a), therefore this dietary information from a single year cannot be applied universally.

Our TAG fatty acid data corresponds well with the findings of Ericson *et al.* (2018a) which reported the fatty acid composition of the total lipid for these 2016 samples. This confirms that the fatty acid composition from the total lipid (TAG + PL) and TAG lipid class are similar, and can both be used to make inferences about krill diet.

The fatty acid composition of the PL fraction of krill also displayed some seasonal variation, particularly for the biomarkers 18:4n-3 and ∑ 20:1 + 22:1. Percentages of 18:4n-3 in PL followed the same trend as TAG, with percentages decreasing as the seasons progressed from summer to winter/spring. Percentages of ∑ 20:1 + 22:1 isomers in PL also showed a similar trend to TAG, but the increased proportion in autumn was more pronounced in PL.

Our study is the first to identify an inconsistent relationship in the TAG/PL ratio of 18:4n-3 in krill. We were able to detect this inconsistent relationship because our study, unlike others, allowed comparisons between all seasons. Most studies with more limited or no seasonal comparisons find that 18:4n-3 is higher in the TAG fraction of krill (Clarke 1980; Falk-Petersen *et al.* 2000; Stübing *et al.* 2003), and other euphausiid species (Kattner & Hagen 1998; Mayzaud *et al.* 1999; Virtue *et al.* 2000) and this has justified its suitability as a trophic marker (Hagen *et al.* 2001). We found that this TAG/PL relationship is not consistent between studies, and that 18:4n-3 may be found in equal or higher proportions in PL during some seasons. This suggests that 18:4n-3 may also be conserved or mobilised to the PL fraction of krill under certain conditions.

Some marine invertebrates are able to elongate and desaturate C18n-3 PUFA to synthesize long chain omega 3 PUFA such as 20:5n-3 and 22:6n-3 (Monroig *et al.* 2013). This has been demonstrated in marine crustaceans (Bell *et al.* 2007) and implies that 20:5n-3 and 22:6n-3 may not solely have a dietary origin. Virtue *et al.* (1993a) suggested that krill may use this elongation-desaturation pathway, as krill fed diets low in 20:5n-3 in the laboratory still had

high percentages of this fatty acid. Levels of 20:5n-3 and 22:6n-3 in krill are also often higher than what could be obtained from the diet alone (Virtue *et al.* 1993b). Bell *et al.* (2007) found that larval krill fed an 18:3n-3 tracer had a very limited ability to synthesize long chain PUFA, and concluded that this may not be a pathway that is readily utilised by krill. Larvae in this study already had sufficient levels of 20:5n-3 and 22:6n-3, however, which may have suppressed synthesis of these fatty acids from the tracer (Bell *et al.* 2007). We hypothesize that 18:4n-3 could be mobilised to PL of krill for conversion to 20:5n-3, and possibly 22:6n-3, during seasons when these important membrane lipids are less available from the diet. A study on an Antarctic copepod species *Calanus propinquus* postulated that such lipid biosynthesis could explain decreased levels of 16:1n-7 and 18:4n-3 in these copepods (Kattner & Hagen 1995). Such mobilisation of 18:4n-3 to PL for conversion to long chain PUFA could explain why percentages of 18:4n-3 did not differ between the TAG and PL of krill in autumn and winter/spring, and why inconsistent TAG/PL ratios of 18:4n-3 are found in different studies. While 18:4n-3 may still be a useful biomarker in krill, it may be modified in TAG and not just replaced by other dietary fatty acids. Further studies are needed to confirm to what extent adult krill are able to carry out conversion of 18:4n-3 to long chain PUFA.

The increased PL percentages of $\sum$ 20:1 + 22:1 in krill our study is also an unusual finding, as previous studies on krill (Clarke 1980; Fricke *et al.* 1984) and other euphausiids (Virtue *et al.* 2000) have found higher percentages in TAG. When the individual fatty acids with 20:1 and 22:1 isomers are separated, the fatty acids 20:1n-9c and 20:1n-7c are higher in the TAG fraction, but 22:1n-9c is higher in the PL fraction. The fatty acid 22:1n-9c was present in proportions of up to 2.7% in krill, which skewed the ratio of TAG/PL towards higher PL composition for this biomarker. It is unknown why 22:1n-9c is higher in the PL, but it may also be the product of elongation-desaturation pathways as it is in copepods (Kattner and Hagen 1995). Differences in percentages of 18:4n-3 and ratios of $\sum$ 20:1 + 22:1 in the TAG and PL

of krill may also relate to whether a study separates the phospholipids into separate phosphatidyl-choline and phosphatidyl-ethanolamine fractions.

The four sterols identified in krill were cholesterol, desmosterol, brassicasterol, and trans-22-dehydrocholesterol. Crustaceans must obtain sterols from the diet as they lack the ability to synthesize them (Kanazawa 2001; Martin-Creuzburg & von Elert 2009). Sterols have a range of roles in organisms as essential constituents of cell membranes, precursors for moulting hormones and as important components for egg production and development (Martin-Creuzburg & von Elert 2009). Cholesterol was the major sterol in krill and appeared to drive the overall sterol composition, which is consistent with the findings of previous studies (Fricke *et al.* 1984; Virtue *et al.* 1993a, 1993b; Phleger *et al.* 2002; Ju & Harvey 2004).

Our study is the first to document seasonal variations in the sterol composition of krill. We cannot establish from this study why krill had significantly higher percentages of cholesterol in winter, but it may relate to seasonally driven physiological processes. Organisms possess the ability to store cholesterol and may use these stores during periods when cholesterol levels are low (Martin-Creuzburg & von Elert 2009). Krill might store cholesterol during the winter months when moulting is less frequent and they undergo reproductive rest. Stored cholesterol may be depleted during late spring, summer and autumn when krill growth rates increase and they are reproducing, then replenished and stored the following winter. Further research is needed to investigate potential storage mechanisms for sterols in krill.

One previous study has also identified only four sterols in krill (Virtue *et al.* 1993a). Others have identified additional algal sterols in krill such as the flagellate marker dinosterol (Virtue *et al.* 1996), and campesterol, stigmasterol, sitosterol, isofucosterol (Virtue *et al.* 1993b, 1996), cholestanol and brassicastanol (Phleger *et al.* 2002). Although sterols must be obtained from the diet, crustaceans may be able to convert a range of these sterols to cholesterol (Virtue

et al. 1993a; Kanazawa 2001; Phleger *et al.* 2002; Martin-Creuzburg & von Elert 2009). This may explain why minor sterols were largely absent from the sterol profile of krill in this study, if they were being dealkylated to desmosterol or cholesterol.

The identified sterols, however, do provide some inferences about the krill diet. Brassicasterol has been found in high quantities in *Phaeocystis pouchetii* (now *Phaeocystis antarctica*) (Nichols *et al.* 1991), therefore, this prymnesiophyte may have been present in the diet of krill particularly during autumn. However, it should also be noted that *P.antarctica* is rich in the fatty acids 14:0, 16:0, 18:0, and 18:1n-9c (Nichols *et al.* 1991; Virtue *et al.* 1993a) and these fatty acids were not significantly higher in krill in autumn compared with the other seasons.

In addition to its role as a precursor to cholesterol (Fricke *et al.* 1984; Virtue *et al.* 1993b), desmosterol is a major component of sterols in diatom ice algae (Barrett *et al.* 1995; Ju & Harvey 2004). Desmosterol percentages were highest in krill which were caught at the WAP and SOI in summer and autumn (regions with sea ice), and lowest in krill caught in winter/spring at SG (permanently ice-free). Higher percentages of desmosterol in summer and autumn krill may be explained by addition of ice-algae in the diet, and the physiological relationship of desmosterol-cholesterol described above.

Our study provides a detailed seasonal description on the composition of fatty acids in the TAG and PL fractions of krill. For the first time, we report variation in TAG and PL fatty acids and sterols across all seasons, and demonstrate that the relationship between specific biomarkers and the lipid classes is more complex than previously thought. Further studies are needed to investigate the ability of krill to synthesize key sterols and fatty acids from other derivatives, and how this synthesis may relate to seasonal fatty acid and sterol composition.

3.6. Acknowledgements

We would like to extend our warmest gratitude to the Captain and crew of Aker Biomarine's FV *Saga Sea* for collecting, carefully packaging and storing the krill used for this study, so as to maintain premium sample integrity. We also thank Dr Andy Revill for facilitating this research, and Mina Brock for technical assistance in the laboratory. This research was funded by Australian Research Council Linkage Grant LP140100412 between the Australian Antarctic Division, Commonwealth Scientific and Industrial Research Organization, Institute for Marine and Antarctic Studies (University of Tasmania), Griffith University and Aker Biomarine.

4 Adult Antarctic krill proves resilient in a simulated high CO_2 ocean

This chapter has been published:

Ericson JA, Hellessey N, Kawaguchi S, Nicol S, Nichols PD, Hoem N, Virtue P (2018). Adult Antarctic krill proves resilient in a simulated high CO_2 ocean. *Communications Biology* **1:** 190. doi: 10.1038/s42003-018-0195-3.

4.1. Abstract

Antarctic krill (*Euphausia superba*) have a keystone role in the Southern Ocean, as the primary prey of Antarctic predators. Any decreases in krill abundance could result in a major ecological regime shift, but there is currently limited information on how climate change may affect krill. Increasing anthropogenic carbon dioxide (CO_2) emissions are causing ocean acidification, as absorption of atmospheric CO_2 in seawater alters ocean chemistry. Ocean acidification increases mortality and negatively affects physiological functioning in some marine invertebrates, and is predicted to occur most rapidly at high latitudes. Here we show that, in the laboratory, adult krill are able to survive, grow, store fat, mature, and maintain respiration rates when exposed to near-future ocean acidification (1000 – 2000 μatm pCO_2) for one year. Despite differences in seawater pCO_2 incubation conditions, adult krill are able to actively maintain the acid-base balance of their body fluids in near-future pCO_2, which enhances their resilience to ocean acidification.

4.2. Introduction

Increasing anthropogenic carbon dioxide (CO_2) emissions are causing atmospheric CO_2 concentrations to rise at a rate unprecedented for millions of years (Doney & Schimel 2007). The global ocean acts as a buffer for rising atmospheric CO_2 levels, as CO_2 is sequestered in the surface waters. This absorption of CO_2 at the air-ocean interface makes seawater more acidic (ocean acidification), due to an increase in the partial pressure of carbon dioxide (pCO_2), hydrogen ions and carbonic acid in seawater (Doney *et al.* 2009). The atmospheric CO_2 concentration has increased by 120 μatm since the industrial revolution (ca. 1850), causing a 0.1 pH unit drop in ocean surface waters (Brewer 2009; Hoegh-Guldberg & Bruno 2010). Model projections suggest that if anthropogenic emissions are not reduced this will result in a further decrease of 0.3 – 0.5 pH units by the year 2100, and 0.77 units by 2300 (Caldeira & Wickett 2003; Ciais *et al.* 2013).

Ocean acidification has negative effects on some marine organisms, causing decreased mineralization or dissolution of calcium carbonate shells, decreased or delayed growth, increased mortality and delayed reproduction or abnormalities in offspring (Kroeker *et al.* 2010). Ocean acidification also causes an increase in pCO_2 (and decrease in pH) in the intra- and extra- cellular spaces of marine organisms, as CO_2 diffuses across cell membranes (Pörtner *et al.* 2004; Melzner *et al.* 2009; Wittmann & Pörtner 2013). The acid-base balance of extracellular fluids must be kept within a certain range for animals to carry out important biochemical functions (Whiteley 2011), prevent metabolic depression and transport oxygen around the body (Wittmann & Pörtner 2013). Despite this range of negative effects, animal responses to acidification are species-specific and a range of positive, negative and neutral responses have been observed in organisms exposed to increased seawater pCO_2 in the laboratory (Harvey *et al.* 2013; Wittmann & Pörtner 2013). Active crustaceans may be more resilient to ocean acidification than other taxonomic groups, due to their increased ability to

regulate extracellular pH (pH_e) compared with more sessile taxa (Melzner *et al.* 2009; Whiteley *et al.* 2011).

Euphausia superba (Antarctic krill, hereafter krill) is the primary prey of marine mammals, penguins and seabirds in the Southern Ocean (Trathan & Hill 2016), which makes it a keystone species in this region. Krill are also the target of the region's largest fishery (Nicol *et al.* 2012). They are highly active crustaceans, and their ability to exploit their environment makes them one of the most abundant organisms on Earth (Tarling & Fielding 2016).

The Southern Ocean is a major carbon sink (Munro *et al.* 2015) and predictions suggest that ocean acidification will occur most rapidly in this region (Hauri *et al.* 2016). Seawater pH in the Southern Ocean varies with season (pH is lower in winter than summer; McNeil & Matear 2008), and pCO_2 is highest at intermediate depths (Kawaguchi *et al.* 2011, 2013). Early life stages of krill (eggs, embryos and larvae) sink to 700 – 1000 m depths during their development before migrating back to surface waters (Quetin & Ross 1984), and adult krill have been found as deep as 3500 m (Clarke & Tyler 2008). Therefore, they may already be exposed to pCO_2 levels up to 550 µatm during their life cycle (Kawaguchi *et al.* 2011). Model projections have shown that the Weddell Sea may reach 1000 µatm pCO_2 at the surface, and 2000 µatm pCO_2 at depth, within the next 80 years (Kawaguchi *et al.* 2013).

Previous short-term studies indicate that Antarctic krill may be more vulnerable to ocean acidification than crustaceans from lower latitudes. Krill eggs fail to hatch at pCO_2 levels predicted to occur by the year 2300 (Kawaguchi *et al.* 2011, 2013), adults increase feeding and nutrient excretion at 750 µatm pCO_2 (Saba *et al.* 2012), and krill may not have the behavioural ability to discriminate between low pCO_2 and high pCO_2 seawater (Yang *et al.* 2018).

Understanding how organisms will respond to high CO_2 requires laboratory experiments that measure a wide range of physiological performance indicators over periods

of months or years (Melzner *et al.* 2009; Whiteley 2011; Meyer & Teschke 2016). To our knowledge, we conducted the first long-term laboratory study to investigate the effects of ocean acidification on adult Antarctic krill. Adult krill were reared for a 46-week period that encompassed all four seasons (25th January – 12th December 2016). Krill were reared in present day seawater pCO_2 concentrations (400 µatm pCO_2, the control), a range of seawater pCO_2 levels predicted to occur in their habitat within the next 100 – 300 years (1000 – 2000 µatm pCO_2), and an extreme level of 4000 µatm pCO_2. Throughout the 46-week experiment we measured a suite of physiological and biochemical variables, to investigate how future ocean acidification may affect the survival, size (total length), lipid stores (triacylglycerol), reproduction (maturity and female ovarian development), metabolism (respiration rate) and extracellular fluid (haemolymph pH) of krill. We show that these physiological processes in krill are largely unaffected by pCO_2 levels predicted within the next 100 – 300 years. Adult krill are able to actively maintain their extracellular pH in 400 – 2000 µatm pCO_2, which enhances their resilience to ocean acidification.

4.3. Materials and Methods

4.3.1. Experimental conditions

Live krill were collected on the RSV *Aurora Australis* via rectangular mid-water trawl on 22nd – 23rd February 2015 (66-03°S, 59-25°E and 66-33°S, 59-35°E). Krill were held in shipboard aquaria using standard maintenance methods (King *et al.* 2003) before being transferred to the Australian Antarctic Division's (AAD) Krill Aquarium in Tasmania (seawater temperature 0.5°C and pH 8.1). Seawater was supplied to aquarium tanks via a seawater recirculating system (Kawaguchi *et al.* 2010).

For ocean acidification experiments, 0.5°C seawater was supplied from a 70 L header tank and equilibrated with air (control) or CO_2-enriched air (elevated pCO_2 treatments) before delivery to experimental tanks. The CO_2-enriched air was monitored using mass flow controllers (Horiba STEC SEC-E-40) and air valves, to regulate flow rates of atmospheric air and pure CO_2. Five experimental 300 L tanks were maintained at five pCO_2 levels; control 400 µatm pCO_2 (pH 8.1), 1000 µatm pCO_2 (pH 7.8), 1500 µatm pCO_2 (pH 7.6), 2000 µatm pCO_2 (pH 7.4) and 4000 µatm pCO_2 (pH 7.1).

Appropriate tank size and the best possible animal husbandry were high priorities in such a long-term study. As krill are a pelagic species, large sized (300 L) experimental tanks were needed to emulate wild conditions as closely as possible in a laboratory. Our experimental design was limited by the space and resources needed for these large tanks, and our observational units (CO_2 treatment tanks) could not be replicated. We did not however, observe any visual evidence to suggest that 'tank' effects were confounding our results.

Two hundred krill were randomly assigned to each experimental tank on 25th January 2016, corresponding to a density of 0.6 individuals L^{-1}. This density is in the range of 0.5 – 2 individuals L^{-1} which has been successfully used in previous experiments at the AAD krill aquarium (Brown *et al.* 2013; Höring *et al.* 2018). The experiment ran for 46 weeks from the 25th Jan 2016 – 12th Dec 2016 covering all four seasons. Mortality rates in all pCO_2 treatments (ranging from 0.03 – 0.2 % day^{-1}) were much lower than previously reported for Antarctic krill in shipboard aquaria (2% day^{-1}; King *et al.* 2003) and in other pCO_2 studies on Pacific krill (0.5 % day^{-1}; Cooper *et al.* 2017) and northern Atlantic krill (5% day^{-1}; Sperfeld *et al.* 2014).

The pCO_2 levels of the CO_2-enriched air and seawater were monitored daily using a LI820 CO_2 gas analyzer and associated computer software (version 2.0.0), and daily pH levels of experimental tanks were measured manually using a pH meter (Mettler Toledo SevenGo Duo

Pro). A three-point calibration of the pH meter was undertaken daily using Radiometer Analytical IUPAC Standard buffers of pH 7.000, 7.413 and 9.180. Total alkalinity (A_T) and dissolved inorganic carbon (DIC) were measured weekly using a Kimoto ATT-05 Total Alkalinity Titrator. Salinity was measured weekly using a Profiline™ Cond 197i Conductivity Meter, WTW. The average total pH (pH_T), pCO_2, calcite and aragonite saturation (Ω_C and Ω_A) values over the 46 week experiment were calculated in CO_2SYS (Pierrot *et al.* 2006) using our measured salinity, temperature, alkalinity and DIC data, and using equilibrium constants of Merhbach, as modified by Dickson and Millero (Dickson *et al.* 2007). Average levels of pCO_2 were 8 – 169 μatm below target levels for the 400 – 2000 μatm treatments, and 123 μatm above the target level for the 4000 μatm treatment. Seawater temperature and A_T were stable in all treatments, while DIC increased with increasing pCO_2. Seawater chemistry in the experimental aquarium is shown in detail in Appendix V.

Krill were fed six days per week with a mixed microalgal diet of the Antarctic flagellate *Pyramimonas gelidicola* at a final concentration of 2 x 10^4 cells ml^{-1}, and Reed Mariculture Inc. (USA) cultures of the diatom *Thalassiosira weissflogii* (8.8 x 10^3 cells ml^{-1}), flagellate *Pavlova lutheri* (4.5 x 10^4 cells ml^{-1}) and flagellate *Isochrysis galbana* (5.5 x 10^4 cells ml^{-1}) (Brown *et al.* 2010; Höring *et al.* 2018).

Light was controlled in the laboratory to ensure that the photoperiod mimicked the seasonally changing light regime of the Southern Ocean (66°S, 30 m depth). Photoperiod was altered monthly, with a maximum of 100 lux light intensity in February and minimum intensity (24 hr darkness) in August (Appendix VI). Light was provided by twin fluorescent tubes and was controlled via standard aquarium procedures (Kawaguchi *et al.* 2010).

4.3.2. Survival

Each pCO_2 treatment was checked daily for mortalities, which were recorded and placed in vials of 10% formalin. Daily mortality data were used to calculate the percentage of krill still surviving at the end of each experimental week in each treatment using the equation:

$$Percentage\ of\ krill\ remaining\ in\ the\ previous\ week - \frac{Number\ of\ mortalities\ during\ the\ current\ week}{Number\ of\ krill\ remaining\ in\ tank} \times 100$$

Krill that were sampled for experimental purposes were not counted as mortalities, but were subtracted from the number of krill remaining in the tank each week. This ensured that the remaining number of krill used to calculate survival percentages reflected actual experimental mortality.

4.3.3. Total length

Krill lengths (mm) were obtained from krill in each pCO_2 treatment in weeks 1, 2, 4, 5, 26, 39, 41, 43 and 46. Sample sizes (*n*) for length measurements for each week and treatment are shown in Appendix VIIA. Individuals were sexed using microscopy and the length of each specimen was measured from the tip of the rostrum to the tip of the uropod (measurement Standard Length 1; Kirkwood 1984). Length data from frozen krill and live krill were combined.

4.3.4. Lipid class analysis (triacylglycerols)

Lipid analysis focused on triacylglycerols which are the main storage fat in krill and, therefore, drive overall lipid concentrations and lipid class composition of krill (Hellessey *et al.* 2018).

Krill were sampled for lipid analysis from all pCO_2 treatments in weeks 1, 2, 4, 5, 26, 39, 41 and 43. Individual krill were placed in cryo-tubes and immediately stored in a –80°C freezer.

Lipid class analysis was carried out on 4 – 5 krill from pCO_2 treatments 400, 1000, 1500 and 2000 on each sampling week (n = 3 for the 4000 µatm tank in weeks 39, 41, and 43 due to increased mortality and lower numbers of krill in that treatment). Sample sizes (n) for each week and treatment are shown in Appendix VIIB. The wet mass (g), total length (measurement Standard Length 1; Kirkwood 1984), and sex for each krill was obtained, and krill were kept frozen during this process to prevent sample degradation. A dry mass (g DM) was obtained later by multiplying the wet mass by 0.2278 to account for the 77.2% water content in the organism (Virtue *et al.* 1993a). Total lipid extracts of krill specimens were obtained using a modified Bligh and Dyer method (Bligh & Dyer 1959; Ericson *et al.* 2018a). Lipid class composition and content were determined using an Iatroscan MK-5 TLC/FID Analyser using standard methods (Hellessey *et al.* 2018).

4.3.5. Sexual maturation

The maturity stages of individual krill were identified during weeks 39, 41, 43 and 46 (n = 5 for 400 – 2000 µatm pCO_2 treatments, n = 3 for the 4000 µatm pCO_2 treatment). Adult krill undergo sexual regression in winter, so these measurements occurred at the end of the experiment to capture the onset of maturity during late spring/early summer.

The sex and maturity stage of each krill was identified via microscopy (using the staging key in Appendix VIII). Each maturity stage was given a maturity score with higher numbers denoting greater maturity (Appendix VIII). After staging, individual krill were placed in a cryopreservation tube with 10% formalin.

4.3.6. Ovarian development

On the final day of the experiment (12th Dec 2016, Week 46), krill left in each experimental tank were preserved in 10% formalin. These samples were used to determine the ovarian development of eight randomly selected females from each of the 400, 1000, 1500 and 2000 µatm pCO_2 treatments. Only two females remained in the 4000 µatm pCO_2 treatment, therefore only two replicates could be obtained for this tank.

The ovary was dissected out of each organism and a single lobe was placed on a microscope slide with a drop of distilled and deionized water and lightly squashed (Cuzin-Roudy & Amsler 1991). Photographs were taken of the ovary section and the lengths of the largest cells (across the longest axis of the cell) were measured using the computer software Image J (https://imagej.nih.gov/ij/). The cell size and photographs were used to determine the maturation stage of krill ovaries using the key in Appendix IX (modified from Cuzin-Roudy & Amsler 1991). When an ovary was transitioning from one stage to another, a 0.5 value was used (e.g. 4.5). Photographic examples of different ovary stages are shown in Appendix X.

4.3.7. Respiration rate

Respirometry measurements were carried out in experimental week 38. Respirometry vessels (2L) with pre-fitted O_2 mini sensors were filled with seawater sourced from the inlet hose of each experimental tank and placed in a 0°C water bath. Each vessel was connected to O_2 computer software (version OXY10v3_50TX) via an optic fiber probe.

Ten krill were sampled from each experimental tank (n = 50 total) and the total length (measurement Standard Length 1; Kirkwood 1984) and wet mass (g) were obtained for each individual. A dry mass (g DM) was obtained by multiplying the wet mass by 0.2278 to account for the 77.2% water content in the organism (Virtue *et al.* 1993a).

Each krill was then placed into a respirometry vessel completely filled with experimental seawater, with no air spaces in the vessels. Oxygen saturation (%) was logged at 5 min intervals in each respirometry vessel over 22-hrs (9AM – 7AM the following day), using the computer software. The software was calibrated at 0°C and the atmospheric pressure at the time of measurement. After 22-hrs krill were removed from the vessels and returned to their experimental tanks.

Only measurements of O_2 saturation (%) taken between 12PM – 7AM were considered for analysis, to ensure that krill had three hours at the beginning of respiration trials to settle into a normal rhythm of respiration before data was collected. Oxygen saturation (100 %) for seawater at 0ºC and 35.1 salinity units (‰) was converted to O_2 ml L^{-1} using the equation in Fox (1907) to obtain a value of 8.035 ml L^{-1}. This was used to convert the O_2 saturation (%) at each logged time point to milliliters of O_2 (O_2 ml) in each 2L respirometry vessel using the equation:

$$O_2 \text{ ml in respirometry vessel} = \frac{\%\ O_2 \text{ saturation}}{100} \times (8.035 \times 2)$$

Values for O_2 ml in each respirometry vessel between 12PM and 7AM were used to create regression equations which were used to compute the O_2 ml used in each respirometry vessel during this period. This value was divided by the krill dry mass (mg), converted to µl O_2 mg DM^{-1}, then divided by 19 hrs to obtain the µl O_2 mg DM hr^{-1}.

4.3.8. Haemolymph pH

The haemolymph pH of five krill from each experimental tank was measured in week 46. Haemolymph pH was measured *in situ* by inserting a pH microelectrode directly into the pericardial cavity. This ensured that air contact with the haemolymph was minimised, as contact with air may alter the CO_2 concentration and pH of the body fluids (Riebesell *et al.* 2011). A Unisense pH Microelectrode (model pH-50, tip diameter 40 – 60 µm) and Unisense Reference Electrode connected to a Unisense pH/mV Meter and computer software (SensorTrace Logger) were used to complete measurements. The pH microelectrode and reference electrode were calibrated using the SensorTrace software via a three-point calibration using Radiometer Analytical IUPAC Standard pH buffers 7.000, 7.413 and 9.180. The buffers were chilled to the seawater temperature in which haemolymph measurements were conducted (0 – 0.5°C). The pH of these buffers at 0°C were used for calibrations (pH 7.12, 7.53 and 9.46 respectively).

Krill were individually removed from their 300 L tanks and placed under a compound microscope in a refrigerated microscope stage, submerged in seawater from the tank they originated from. The pH of this seawater was measured using the microprobe and reference probe, and a portable pH meter (Mettler Toledo SevenGo Duo Pro) to ensure that the measurements matched to within < 0.05 pH units before proceeding.

Live krill were restrained within the microscope stage using acrylic blocks, designed to expose the integument that links the krill carapace to the abdomen. A micromanipulator was used to position the microelectrode relative to the animal. A camera connected to the compound microscope was also used to magnify the krill carapace-abdomen joint and view the real-time image on a computer monitor to ensure the accuracy of microelectrode placement.

The microelectrode was inserted through the integument underneath the carapace and into the pericardial cavity between the thorax and first abdominal segment. The reference probe remained in the seawater surrounding the krill during this process. Some resistance was observed as the microelectrode pierced the integument, causing a slight tear in the body wall as the probe penetrated the integument, ensuring that electrical conductivity was maintained between the reference probe and microelectrode.

The SensorTrace Logger software logged the pH of the haemolymph, and the pH was recorded once the reading had stabilised after approximately 1 min. The microelectrode was then withdrawn from the abdomen and haemolymph was observed leaking into the surrounding seawater as positive pressure from within the animal pushed it outwards. The krill was removed from the microscope stage and preserved in 10% formalin.

4.3.9. Statistical analyses

Data were analysed in the RStudio statistics package (version 0.99.893) using one-way ANOVA with pCO_2 treatment as a factor, or two-way ANOVA with pCO_2 and Week as factors. Dunnett comparisons (carried out using the RStudio 'multcomp' package) were used to identify significant differences between the control treatment (400 μatm pCO_2) and all other factor levels, while Tukey Post-hoc comparisons were used to compare all factor levels with one another. Polynomial contrasts were used to identify linear, quadratic and cubic trends in the data. Type 3 Sums of Squares (SS) were used when data was unbalanced and Type 1 SS were not appropriate. Data were log or square root transformed when assumptions of normality or homogeneity of variances were not met. Data in tables are expressed as mean ± standard deviation. For all analyses, α was set at 0.05 and all tests were two tailed. The RStudio packages 'ggplot2', 'plyr' and 'dplyr' were used to produce all figures.

4.4. Results

4.4.1. Survival

The survival rate of krill was highest in present day and future pCO_2 seawater (400 – 2000 µatm) throughout most of the experiment (Figure 4.1). The survival rate of krill by week 46 was higher in the 1000 – 2000 µatm pCO_2 treatments (87 – 90%) than the control (400 µatm) treatment (79%). Large decreases in survival rate occurred between weeks 3 – 7 and weeks 19 – 22 in the extreme pCO_2 (4000 µatm) treatment and plateaued towards the end of the experimental period (weeks 29 – 46), with 53% of individuals surviving by week 46 (Figure 4.1).

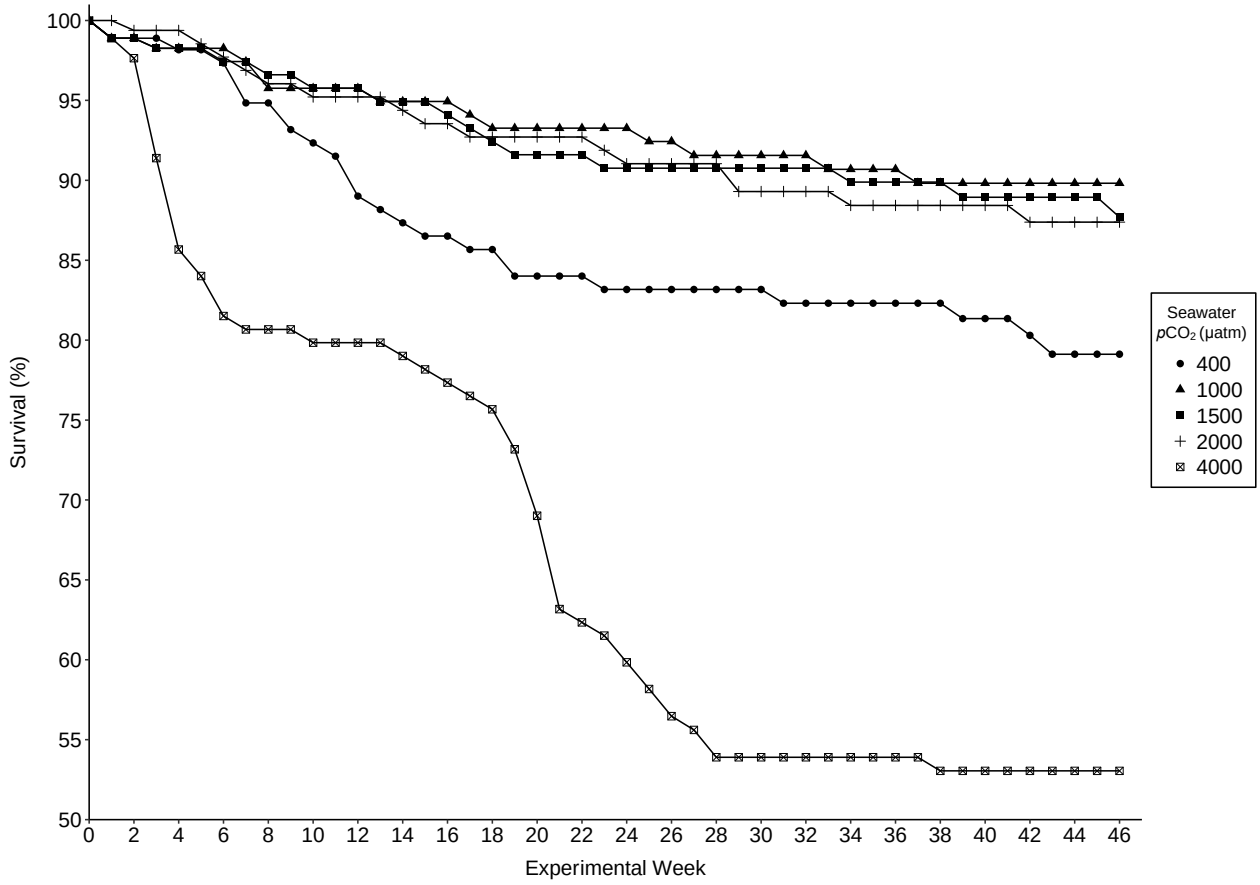


Figure 4.1. Survival (%) of *Euphausia superba* in each experimental week in 400 (present day control), 1000, 1500, 2000 and 4000 µatm pCO_2 seawater.

4.4.2. Body length and triacylglycerol content

Krill in all treatments maintained their total length and triacylglycerol content (fat stores) during summer (weeks 1, 2, 4 and 5; Figures 4.2 and 4.3), with no differences observed between pCO_2 treatments or weeks for length (Two Way ANOVA, pCO_2*week; df = 12, F = 1.12, p = 0.359) or triacylglycerol content (Two Way ANOVA, pCO_2*week; df =12, F = 1.14, p = 0.341).

By winter (week 26), the median length of krill in all treatments had decreased (Figure 4.2). Krill in extreme pCO_2 seawater (4000 µatm) were shorter (Dunnett test, p = 0.023) and had stored less fat (Dunnett test, p = 0.041) than krill in ambient pCO_2 seawater.

Throughout spring, krill in the 4000 µatm pCO_2 treatment were shorter than krill in 400 µatm pCO_2 (Dunnett tests week 39; p = 0.094, week 41; p < 0.001, week 43; p = 0.005), but no differences were seen between treatments by the following early summer (week 46; One Way ANOVA pCO_2; df = 4, F = 0.73, p = 0.584). Triacylglycerols remained lower in krill in the 4000 µatm pCO_2 treatment compared with krill in ambient pCO_2 throughout early spring (Dunnett tests week 39; p = 0.021 and week 41; p < 0.001), but all treatments had similar triacylglycerol content by late spring (week 43; One Way ANOVA pCO_2; df = 4, F = 1.13, p = 0.379).

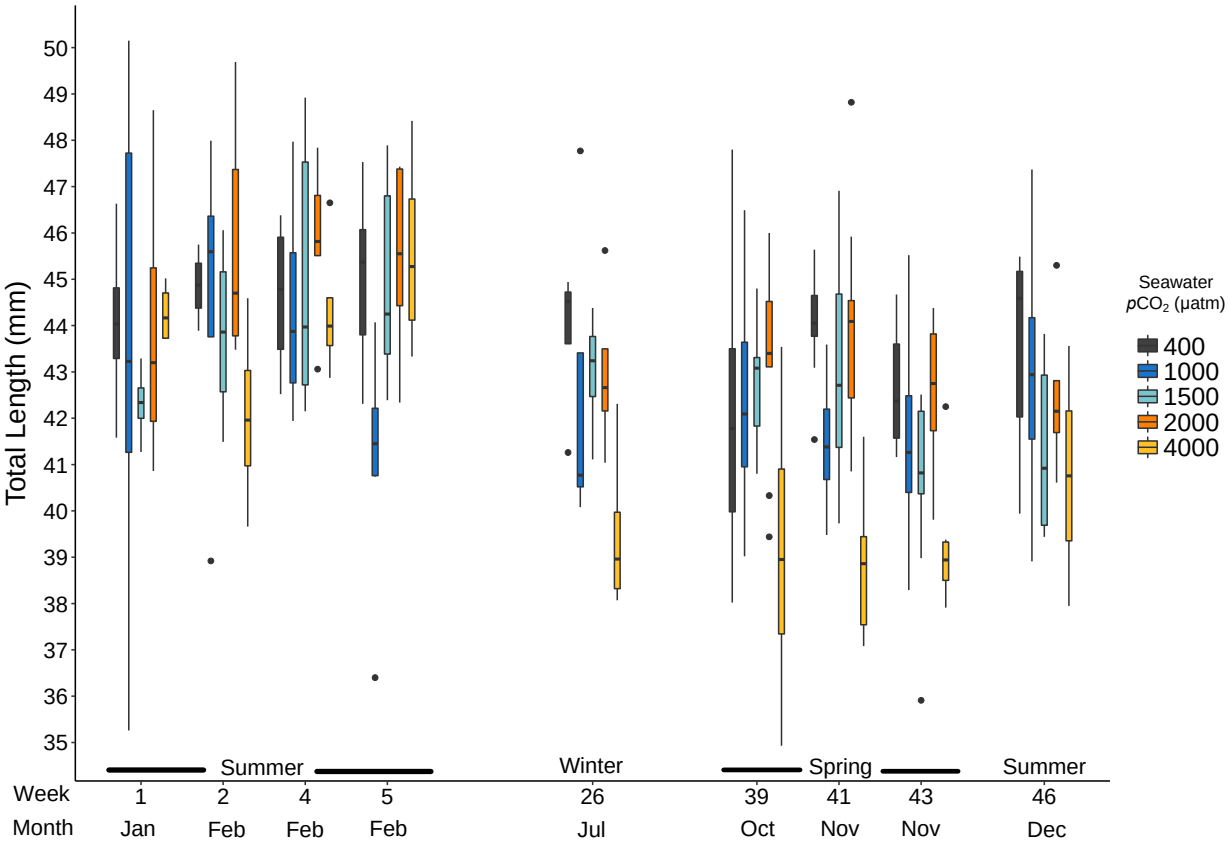


Figure 4.2. Total length (mm) of *Euphausia superba* in 400, 1000, 1500, 2000 and 4000 µatm pCO_2 seawater in weeks 1, 2, 4, 5, 26, 39, 41, 43 and 46 of the experiment. Box plot elements are centre line, median; box limits, upper and lower quartiles; whiskers extend to the most extreme data point no more than 1.5 times the interquartile range. Shaded black dots denote outlier values that are over 1.5 times the interquartile range. Months and seasons corresponding to the experimental weeks are also provided (X-axis). For each pCO_2 by week combination n = 2 – 10. See Appendix VIIA for exact sample sizes for each treatment combination.

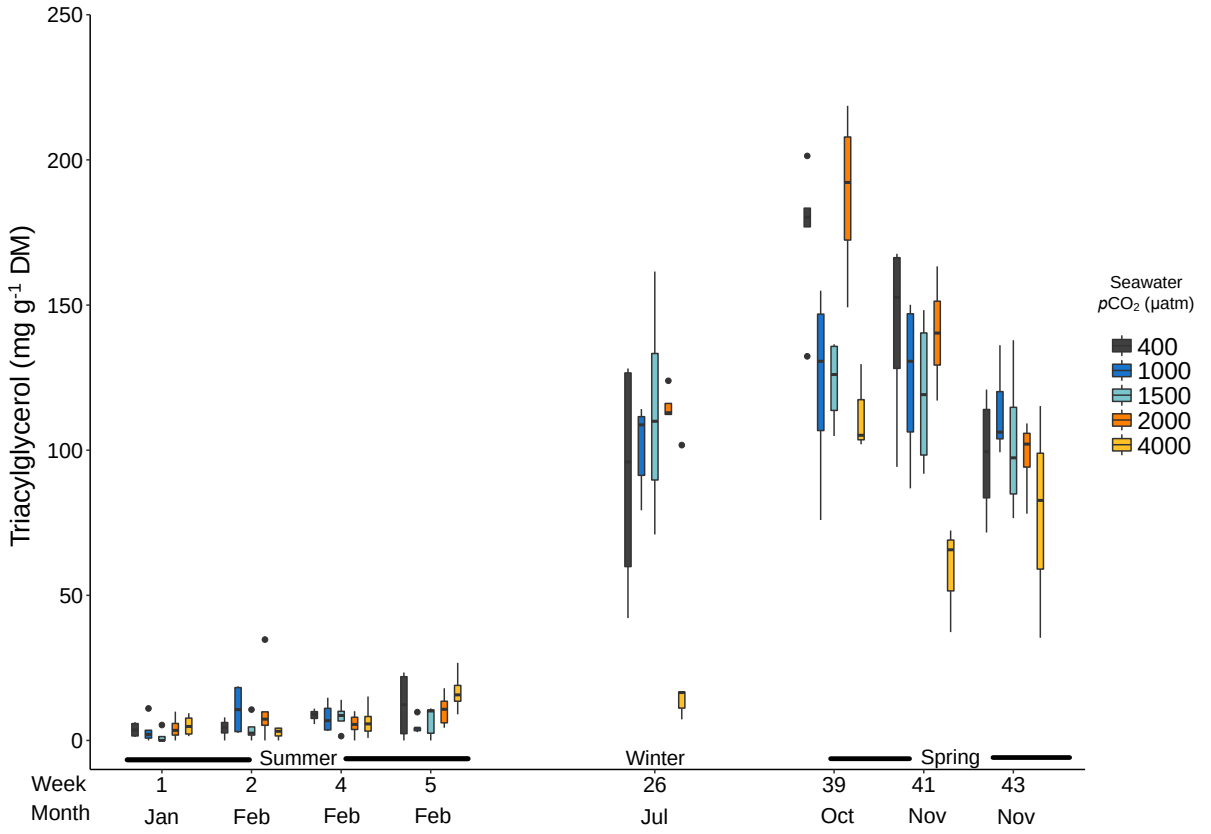


Figure 4.3. Triacylglycerol (mg g^{-1} dry mass; DM) of *Euphausia superba* in 400, 1000, 1500, 2000 and 4000 µatm pCO_2 seawater in weeks 1, 2, 4, 5, 26, 39, 41 and 43 of the experiment. Box plot elements are centre line, median; box limits, upper and lower quartiles; whiskers extend to the most extreme data point no more than 1.5 times the interquartile range. Shaded black dots denote outlier values that are over 1.5 times the interquartile range. Months and seasons corresponding to the experimental weeks are also provided (X-axis). For each pCO_2 by week combination n = 3 – 5. See Appendix VIIB for exact sample sizes for each treatment combination.

4.4.3. Sexual maturation and ovarian development

The sexual maturity of krill in the 400 – 2000 µatm pCO_2 treatments advanced between spring and early summer (weeks 39 – 46) and all krill reached maturity at similar times (Appendix XI). When maturity scores from weeks 39 – 46 were combined, overall maturity scores of krill were lowest in the 4000 µatm pCO_2 treatment, suggesting delayed sexual development (Figure 4.4A). Krill in 400 – 2000 µatm pCO_2 had completed ovarian development to the previtellogenesis or early vitellogenesis stages by week 46, but ovarian development in krill in

the 4000 µatm pCO_2 treatment was delayed and had not progressed past oogenesis (Figure 4.4B).

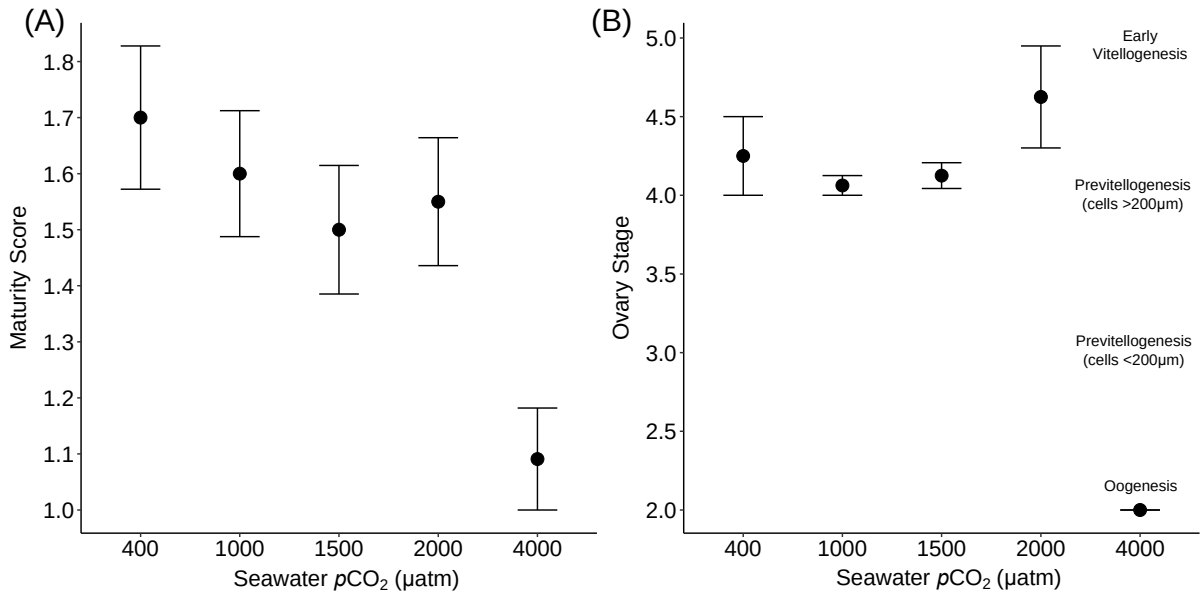


Figure 4.4. Maturity scores (**A**) and ovary stages (**B**) of *Euphausia superba* in 400, 1000, 1500, 2000 and 4000 µatm pCO_2 seawater. Maturity scores are the average scores (mean ± SE) of krill in each treatment for weeks 39, 41, 43 and 46 combined (n = 5 for the 400 – 2000 µatm pCO_2 treatments and n = 3 for the 4000 µatm pCO_2 treatment). Ovary stages are the average ovary stages (mean ± SE) of female krill in week 46 only. The physical ovary stages relating to each stage number are also provided (2 = oogenesis, 3 = previtellogenesis (cell size < 200 µm), 4 = previtellogenesis (cell size > 200 µm), 5 = early vitellogenesis). Higher maturity scores and ovary stages indicate a more advanced reproductive stage. For 400 – 2000 µatm pCO_2 treatments n = 8, and for the 4000 µatm pCO_2 treatment n = 2.

4.4.4. Respiration rate

Respiration rates of krill in early spring (week 38) ranged from 0.13 – 0.50 µl O_2 mg DM hr^{-1} (Figure 4.5) and did not differ between pCO_2 treatments (One Way ANOVA, pCO_2; df = 4, F = 1.26, p = 0.301). Intraspecific variation in the respiration rates of individual krill increased in elevated pCO_2 treatments (Figure 4.5).

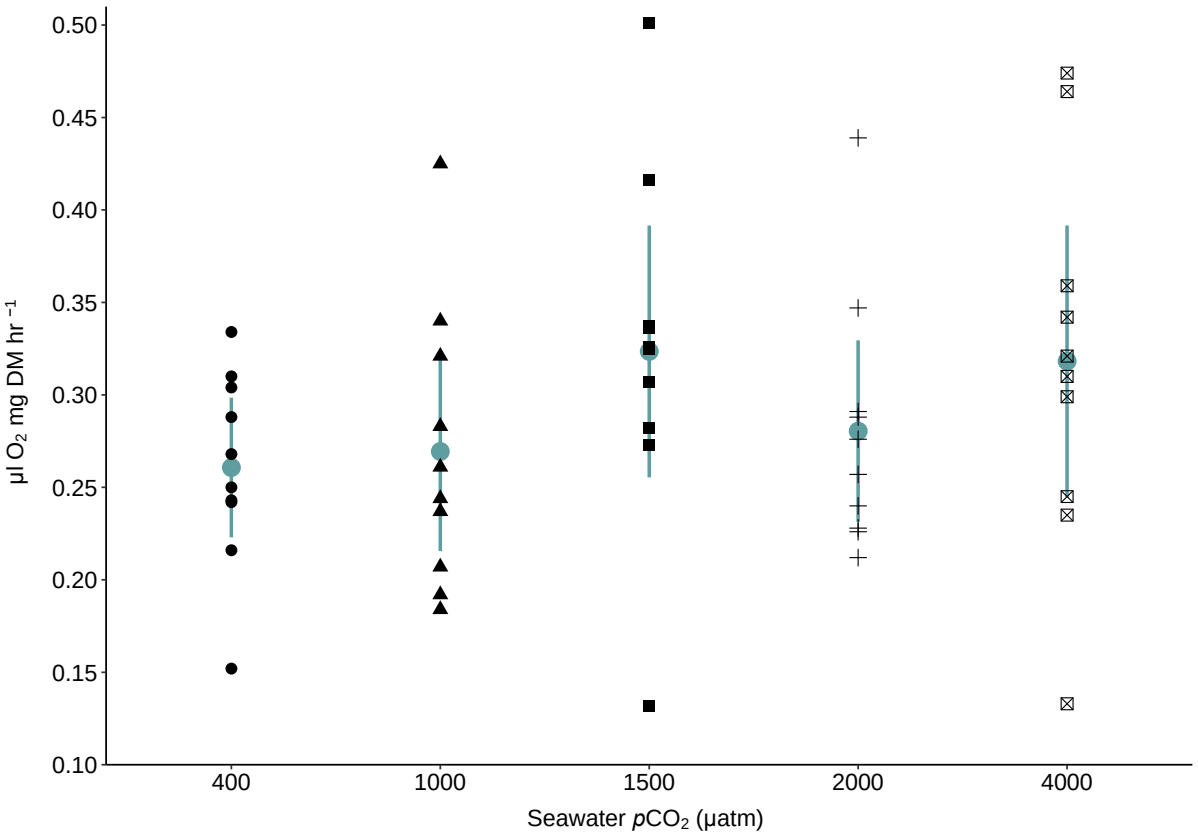


Figure 4.5. Respiration rates (µl O_2 mg dry mass hr^{-1}) of *Euphausia superba* in 400, 1000, 1500, 2000 and 4000 µatm pCO_2 seawater in experimental week 38 (early spring). Each black point denotes the respiration rate of an individual krill (n = 10 for each pCO_2 treatment). Blue circles denote the mean for each pCO_2 treatment, and error bars represent the 95% confidence intervals for the mean.

4.4.5. Haemolymph pH

Haemolymph pH of krill measured in week 46 ranged from pH 7.57 – pH 8.47. Haemolymph pH of krill in 1000 – 2000 µatm pCO_2 treatments did not differ significantly from the control (Dunnett tests; 1000 µatm p = 1.000, 1500 µatm p = 0.145, 2000 µatm p = 0.369) (Figure 4.6). The average haemolymph pH of krill in the 4000 µatm pCO_2 treatment was 0.5 units lower than krill in the control treatment (Dunnett test, $p < 0.001$). There was a linear trend of decreasing haemolymph pH with increasing pCO_2 (One Way ANOVA with polynomial contrasts, pCO_2; df = 4, F = 11.69, linear $p < 0.001$).

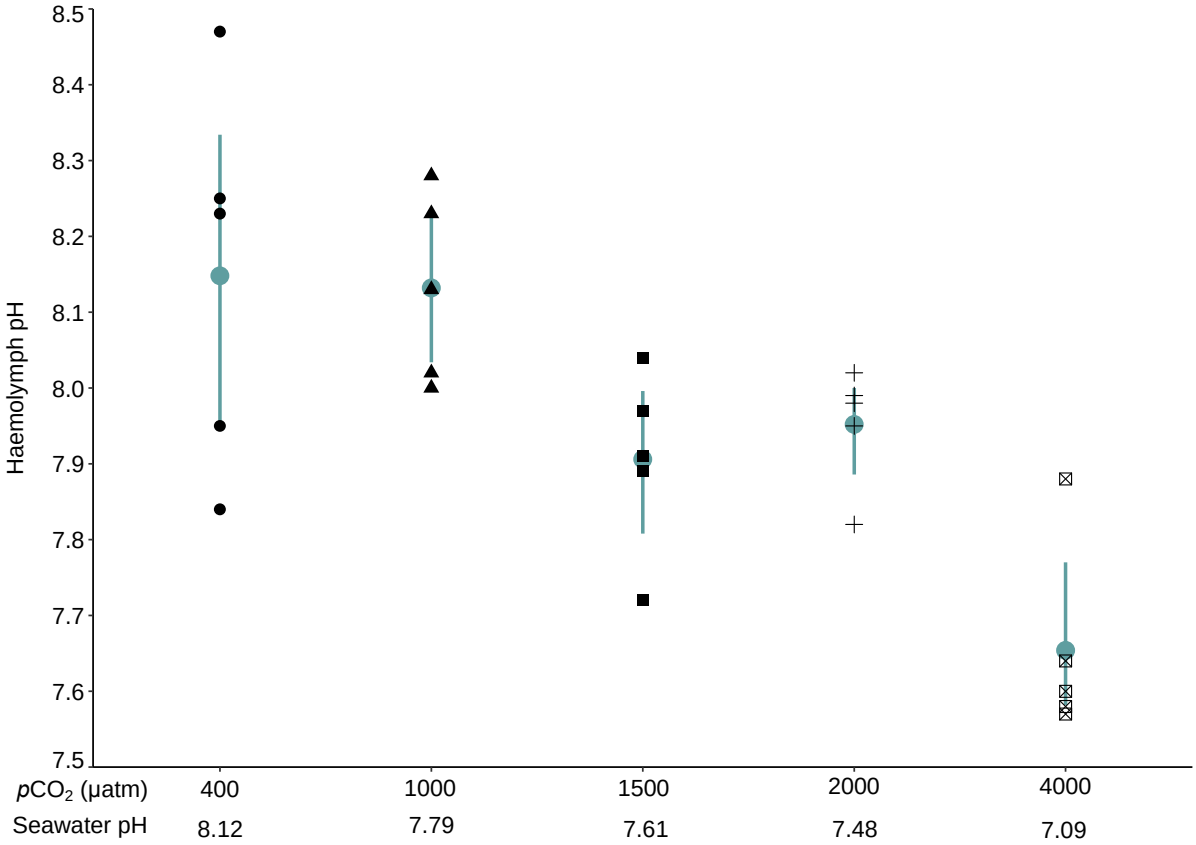


Figure 4.6. Haemolymph pH of *Euphausia superba* in 400, 1000, 1500, 2000 and 4000 µatm pCO_2 seawater in experimental week 46. Each black point denotes the haemolymph pH of an individual krill (n = 5 for each pCO_2 treatment). Blue circles denote the mean for each pCO_2 treatment, and error bars represent the 95% confidence intervals for the mean. Seawater pH values are shown below pCO_2 treatments for ease of interpretation.

4.5. Discussion

Our experimental results show that the measured physiological processes in adult Antarctic krill were robust to near-future ocean acidification (1000 – 2000 µatm pCO_2), when elevated pCO_2 was assessed as a single stressor. The survival rate of krill subject to near-future pCO_2 increased by up to 11%, and seasonal patterns of growth, fat storage and reproductive development were comparable to wild krill (Meyer *et al.* 2010; Kawaguchi 2016; Hellessey *et al.* 2018). These physiological processes appeared to be controlled by endogenous rhythms

(Meyer 2012; Meyer & Teschke 2016; Höring *et al.* 2018), and were not affected by near-future pCO_2.

Most studies report a decrease in survival when organisms are exposed to acidification (Kroeker *et al.* 2010). In contrast, slight increases in euphausiid survival rates have been observed in *Euphausia pacifica* after a 2-month exposure to 1200 µatm pCO_2 (Cooper *et al.* 2017) and in *Nyctiphanes couchii* after a 35-day exposure to 800 µatm pCO_2 seawater (Sperfeld *et al.* 2014). Euphausiids that are exposed to vertically changing pCO_2 in the water column may use acid-base regulation and short-term metabolic depression (reduced respiration rates) to enhance survival in high pCO_2 conditions (Cooper *et al.* 2016, 2017).

Primary productivity may increase in high pCO_2 seawater, increasing food supply and subsequent survival of herbivores in these experimental treatments (Sswat *et al.* 2018). It is unlikely that phytoplankton growth (and therefore food supply) increased in our high pCO_2 tanks, as phytoplankton was grazed by krill within ~2 hrs. Furthermore, the majority of phytoplankton added to tanks were non-viable cultures that do not photosynthesize. Further targeted studies on krill survival under ocean acidification conditions, and effects of pCO_2 on their food sources, may identify whether krill survival is enhanced in elevated pCO_2 seawater.

In our study, pCO_2 levels between 1000 – 2000 µatm did not affect the size of adult krill over a whole year and this reflects their ability to moult and grow. Reduced growth rates have been observed in adult crustaceans exposed to high pCO_2 seawater for short-medium term durations (weeks to months; Whiteley 2011). Elevated pCO_2 did not affect growth rates in the north Atlantic euphausiids *Nyctiphanes couchii* (Sperfeld *et al.* 2014) or *Thysanoessa inermis* (Opstad *et al.* 2018) after short-term (5 – 11 week) exposure, but exposure to levels of 1200 µatm pCO_2 over 2 months slowed growth in *Euphausia pacifica* (Cooper *et al.* 2017).

Krill in our study were shorter than wild krill which can grow up to 60 mm in total

length (Reiss 2016). Growth of wild krill is closely related to food quality and quantity, and laboratory reared krill do not grow as large as wild krill (Brown *et al.* 2010). It is impossible to directly replicate the wild diet in controlled conditions, therefore the shorter lengths attained by krill in our study may have been caused by lower food quality in the laboratory. Patterns of seasonal growth seen in wild krill (e.g. winter shrinkage) were, however, observed in our experimental krill, suggesting that the experimental conditions replicated the physiological cycle of wild krill as closely as possible.

The resilience of Antarctic krill, in terms of their maturation and ovarian development to near-future pCO_2, is comparable to other pelagic crustaceans. Short-term studies (< 2 weeks) have generally found that egg production is not affected by moderately increased pCO_2 levels (Kurihara *et al.* 2004a; Kurihara & Ishimatsu 2008), but production rates decrease significantly in crustaceans exposed to extreme pCO_2 levels (Kurihara *et al.* 2004b).

Decreased growth and delayed reproduction are often observed in sessile organisms that cannot maintain their acid-base balance, and those that decrease their metabolism when exposed to high pCO_2 (Kroeker *et al.* 2010). This occurs because energy is diverted away from growth and reproduction, and prioritized for acid-base compensation (Whiteley 2011). The ability of active Antarctic krill to maintain their size and mature in 1000 – 2000 μatm pCO_2 is likely to be directly linked to their ability to maintain acid-base balance and respiration rates at these pCO_2 levels.

An increase in krill metabolic activity has been observed after short term (24 hr) exposure to ocean acidification (Saba *et al.* 2012), suggesting that krill may raise their respiration rate on initial exposure to high pCO_2. The increasing variation in krill respiration rates at higher pCO_2 levels suggests that individuals vary in their capacity to respond to CO_2-induced metabolic stress. This may be due to intraspecific differences in phenotypic plasticity,

or genetic predisposition to metabolic resilience in some individuals (Sunday *et al.* 2011; Carter *et al.* 2013).

The ability of krill to maintain their acid-base balance in elevated pCO_2 seawater may be the key to their successful survival, maturity and growth in a future high CO_2 world. Haemolymph pH can be increased in hypercapnic conditions via ion transport pumps that pump bicarbonate into the extracellular space (Henry & Wheatly 1992; Melzner *et al.* 2009). These pumps are located in the gill epithelia and consume energy as they actively transport ions in and out of body compartments (Wittmann & Pörtner 2013). Our results suggest that krill in elevated pCO_2 were actively maintaining haemolymph pH, as it remained within the same range for krill in 400 – 2000 μatm pCO_2. The negligible effects on growth and reproduction in these krill indicate that they were able to actively regulate acid-base balance at low energetic cost. The trend of decreasing haemolymph pH with increasing pCO_2 indicates that although krill in near-future pCO_2 were able to maintain haemolymph pH within the same range as krill in ambient pCO_2, measurements were within the lower range of values for krill in ambient pCO_2. This may have implications for longer term acid-base maintenance. The ability for krill to maintain haemolymph pH beyond one year, and into their spawning season, is unknown. The substantial increase in mortality in extreme pCO_2 (4000 μatm) may have been caused by the inability of those krill to maintain acid-base balance.

Unlike decapods with gills located inside their carapace, Antarctic krill have external gills with a complex structure built for efficient ion and gas exchange (Alberti & Kils 1983). These intricate gills are designed to maximise the amount of O_2 available to krill during swarming and frequent periods of intense swimming activity (Alberti & Kils 1983). The ability to rapidly exchange O_2, CO_2, and ions across their external gills may have assisted krill in maintaining acid-base balance and respiration rates when exposed to near-future acidification.

Modification of the respiratory pigment haemocyanin may also assist crustaceans to maintain levels of O_2 consumption during hypercapnia (Whiteley 2011), however this was not measured in our study.

Krill have evolved a unique range of adaptations to survive the Antarctic winter (Meyer 2012). Metabolic depression is one such adaptation that is controlled endogenously, cued by the seasonal light cycle (Teschke *et al.* 2007; Meyer *et al.* 2010; Brown *et al.* 2013). The physiological responses of krill in our extreme pCO_2 treatment (4000 µatm pCO_2) suggest that this energy-conserving strategy may be less advantageous in high pCO_2 conditions. In winter, as the photoperiod approached 24-hr darkness, krill growth and fat deposition in 4000 µatm pCO_2 seawater were reduced compared with krill in ambient pCO_2. In this extreme environment, metabolic depression during winter may have prevented krill from maintaining respiration rates high enough to maintain pH_e, grow and store fat. These reductions in winter growth and fat storage may have contributed to the subsequent delay in reproductive development (Kawaguchi *et al.* 2007).

The energy needed to maintain pH_e can be met by consuming more food (Li & Gao 2012), and Antarctic krill do increase their feeding rates in elevated pCO_2 seawater (Saba *et al.* 2012). The constant food supply in our experiment may have enabled krill in the 4000 µatm pCO_2 treatment to perform better than if they had received food at seasonally variable concentrations. Importantly, this may have also enabled krill in lower pCO_2 treatments (1000 – 2000 µatm pCO_2) to maintain haemolymph pH, normal growth, and reproductive development. The relationship between food supply and pCO_2 can affect predator physiology in different ways (Brown *et al.*, 2018), and requires further investigation. Metabolic depression, the increasing severity of winter acidification (McNeil & Matear 2008), and regionally variable food concentrations (Schmidt & Atkinson 2016) may increase the vulnerability of krill to near-

future ocean acidification during winter.

The prosperity of Antarctic krill in a high CO_2 world will depend on the ability of adults to produce offspring resilient to ocean acidification. If early life stages cannot survive, this may have catastrophic consequences for krill populations and the Southern Ocean ecosystem. Previous studies indicate that krill eggs and embryos are sensitive to seawater pCO_2 above 1250 μatm (Kawaguchi *et al.* 2011, 2013). These studies used gametes from parents that were maintained in ambient pCO_2 conditions, and gametes were spawned into ambient seawater before being subjected to high pCO_2 conditions. Recent studies have shown that some adult echinoderms and molluscs that acclimate to high pCO_2 conditions are able to produce gametes resilient to high pCO_2 (Suckling *et al.* 2015; Ross *et al.* 2016), and this may allow such species to adapt to ocean acidification over generational time scales (Foo & Byrne 2016). Further studies may establish whether this generational adaptation occurs in krill, which would influence the way that we assess the vulnerability of the early life stages.

Our results suggest that adult Antarctic krill are resilient to ocean acidification, and may not be affected by pCO_2 levels predicted for the next 100 – 300 years. The overall resilience of Antarctic krill as a species will, however, depend on long-term effects occurring at all life history stages. Endogenous rhythms controlling metabolic rate, combined with food availability in the wild, may influence the vulnerability of krill to high pCO_2 in winter. Negative effects on krill physiology may be seen at near-future pCO_2 levels if effects of acidification are exacerbated by other stressors such as ocean warming. The persistence of krill in the Southern Ocean is vital for the health of the Antarctic ecosystem, and we are only just beginning to understand how this keystone species may respond to climate change.

4.6. Acknowledgements

We thank Tasha Waller, Ashley Cooper, Rob King and Blair Smith for technical support in the Krill Aquarium, and Guang Yang for assistance with daily experimental monitoring. We thank Simon Wotherspoon for statistical advice. This research was funded by an Australian Research Council Linkage Grant LP140100412 between the Australian Antarctic Division, Commonwealth Scientific and Industrial Research Organisation, Institute for Marine and Antarctic Studies (University of Tasmania), Aker Biomarine and Griffith University.

5 Near-future ocean acidification does not alter the lipid content and fatty acid composition of adult Antarctic krill

This chapter is under review for *Scientific Reports*:

Ericson JA, Hellessey N, Kawaguchi S, Nichols PD, Nicol S, Hoem N, Virtue P (under review). Near-future ocean acidification does not alter the lipid content and fatty acid composition of adult Antarctic krill.

5.1. Abstract

Antarctic krill (*Euphausia superba*) are a keystone species in the Southern Ocean, but little is known about how they will respond to climate change. Ocean acidification, caused by sequestration of carbon dioxide into ocean surface waters (pCO_2), is known to alter the lipid biochemistry of some organisms. This can have cascading effects up the food chain. In a year-long laboratory experiment adult krill were exposed to ambient seawater pCO_2 levels (400 µatm), elevated pCO_2 levels that mimicked near-future ocean acidification (1000, 1500 and 2000 µatm) and an extreme pCO_2 level (4000 µatm). The laboratory light regime mimicked the seasonal Southern Ocean photoperiod and krill received a constant food supply. Total lipid mass (mg g^{-1} DM) of adult krill was unaffected by near-future levels of seawater pCO_2. Fatty acid composition (%) and fatty acid ratios associated with immune responses and cell membrane fluidity were also unaffected by near-future pCO_2, apart from an increase in 18:3n-3/18:2n-6 ratios in krill in 1500 µatm pCO_2 in winter and spring. Extreme pCO_2 had no effect on krill lipid biochemistry during summer. During winter and spring, krill in extreme pCO_2 had elevated levels of omega-6 fatty acids (up to 1.2% increase in 18:2n-6, up to 0.8% increase in 20:4n-6 and lower 18:3n-3/18:2n-6 and 20:5n-3/20:4n-6 ratios), and showed evidence of increased membrane fluidity (up to three-fold increase in phospholipid/sterol ratios). These results indicate that the lipid biochemistry of adult krill is robust to near-future ocean acidification.

5.2. Introduction

Antarctic krill (*Euphausia superba*, hereafter 'krill') are a highly abundant keystone species in the Southern Ocean food web (Trathan & Hill 2016). Their large biomass and dense swarms make them the primary food source for a range of Antarctic mega-fauna (whales, seals and penguins), fish, squid and seabirds. Krill are lipid-rich and contain high concentrations of nutritious long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFA), particularly eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) (Virtue *et al.*, 1993a; Ericson *et al.* 2018a).

Krill are likely to be affected by anthropogenic climate change as ocean warming, sea level rise, sea ice melt and biological invasions increase (Rintoul *et al.* 2018). Ocean acidification, caused by sequestration of atmospheric CO_2 into ocean surface waters, may be particularly severe in polar marine ecosystems (Andersson *et al.* 2008; Fabry *et al.* 2009; Steinacher *et al.* 2009). As atmospheric CO_2 emissions rise, approximately 30% of these emissions are absorbed into seawater at the air/ocean interface (Doney *et al.* 2009). This increase in seawater pCO_2 alters the chemical equilibrium of seawater, causing a decrease in seawater pH (Raven *et al.* 2005). Average ocean pH has already decreased by 0.1 units since the industrial revolution, and is predicted to decrease by another 0.33 units by the year 2300 if CO_2 emissions are not mediated (Caldeira & Wickett 2003; Ciais *et al.* 2013).

Understanding how species may respond to ocean acidification can be assisted by long-term laboratory experiments which expose organisms to predicted future levels of seawater pCO_2. Investigating how animals regulate their physiology in response to environmental changes can be examined through lipid biochemistry. Lipids and their associated fatty acids have a diverse range of roles in the body; they are indicators of an organism's diet and condition (Dalsgaard *et al.* 2003), and can be used to detect biochemical shifts in response to stress

(Hixson & Arts 2016; Bennett *et al.* 2018). Triacylglycerol (TAG) lipid classes are used for fat storage, while phospholipids (PL) and sterols (ST) make up the structure of cell membranes (Parrish *et al.* 2000). Long chain polyunsaturated fatty acids (LC-PUFA) such as 20:5n-3 (eicosapentaenoic acid) and 22:6n-3 (docosahexaenoic acid) are abundant anti-inflammatory fatty acids in PL membranes and conserved for reproductive processes (Kattner *et al.* 2007; Yoshida *et al.* 2011; Corsolini & Borghesi 2017). Arachidonic acid (20:4n-6) is a less abundant but equally important LC-PUFA, being the primary precursor of eicosanoids, which regulate the immune system, reproductive processes, and ion flux (Stanley-Samuelson 1987). Elevated ratios of n-6/n-3 PUFA in organisms are indicators of inflammation and physiological stress (Calder & Grimble 2002; Van Anholt 2004). Homeoviscous adaptation (regulation of cell membrane fatty acids, which occurs in response to stressors (Ernst *et al.* 2016)), can be investigated by measuring ratios of polyunsaturated/saturated fatty acids (PUFA/SFA), ratios of PL/ST, and mean carbon chain length (MCL) in organisms (Bennett *et al.* 2018). The kinked formation of PUFA increases membrane fluidity, while the absence of double bonds in densely packed SFA increases membrane stability (Nelson & Cox 2012). Sterols can also be incorporated into cell membranes and packed between PUFA to increase membrane thickness (Ernst *et al.* 2016).

Studies have found a range of effects of increased pCO_2 and/or temperature on the lipid biochemistry of marine organisms: phytoplankton (Wynn-Edwards *et al.* 2014; Bermúdez *et al.* 2015; King *et al.* 2015; Sommer *et al.* 2015; Bermúdez *et al.* 2016; Hixson & Arts 2016; Wang *et al.* 2017; Bi *et al.* 2018), sponges (Bennett *et al.* 2018), fish (Díaz-Gil *et al.* 2015; Murray *et al.* 2017), crustaceans (Garzke *et al.* 2016; Gao *et al.* 2018), echinoderms (Matson *et al.* 2012; Verkaik *et al.* 2016), corals (Strahl *et al.* 2016) and molluscs (Valles-Regino *et al.* 2015). Ocean acidification may result in a transition from high- to low-lipid phytoplankton and

zooplankton species, and this could affect the health of higher predators (Kattner *et al.* 2007; Rossoll *et al.* 2012).

Krill lipids have been widely studied due to the importance of krill in the food web (Virtue *et al.* 1993b; Hagen *et al.* 2001; Alonzo *et al.* 2005b; Schmidt & Atkinson 2016; Ericson *et al.* 2018a; Hellessey *et al.* 2018), and commercial interest from the Antarctic krill fishery (Gigliotti *et al.* 2011; Nicol *et al.* 2012). A recently published study showed that adult Antarctic krill are only affected by severe levels of pCO_2 (Ericson *et al.* 2018b), but there is no published information on changes in krill lipid mass and fatty acid composition with elevated pCO_2. It is essential to understand whether krill lipid composition will be affected by climate change because the Southern Ocean ecosystem is largely fuelled by lipid energy derived from krill.

Our study aimed to investigate the resilience of adult krill by observing the effects of elevated pCO_2 on detailed aspects of krill lipid biochemistry. We reared krill for one year (January – December 2016) in ambient pCO_2 levels (400 µatm pCO_2), those predicted for the near-future (within the next 100 – 300 years; 1000, 1500 and 2000 µatm pCO_2), and an extreme level of 4000 µatm pCO_2. We analysed krill samples to observe whether the total lipid and fatty acid composition of krill changed with pCO_2 over this long-term experiment. We also investigated whether lipid indicators of (a) homeoviscous adaptation (PUFA/SFA ratios, PL/ST ratios, and MCL) and (b) immune responses (n-3/n-6; 22:6n-3/20:4n-6 ratios and 18:3n-3/18:2n-6 ratios) in krill were altered by seawater pCO_2.

5.3. Materials and Methods

5.3.1. Experimental conditions

Experimental conditions are described in detail in Ericson *et al.* (2018b). Briefly, krill were collected from the Southern Ocean (66-03°S, 59-25°E and 66-33°S, 59-35°E) on the RSV

Aurora Australis, using a mid-water trawl net. They were held in shipboard aquaria using standard husbandry methods (see King *et al.* 2003) and transported to the Australian Antarctic Division Krill Aquarium in Tasmania.

For ocean acidification experiments, five 300L tanks were equilibrated to five pCO_2 levels; 400 µatm pCO_2 (pH 8.1 control treatment), 1000 µatm pCO_2 (pH 7.8), 1500 µatm pCO_2 (pH 7.6), 2000 µatm pCO_2 (pH 7.4) and 4000 µatm pCO_2 (pH 7.1). Seawater temperature of all tanks was held at 0.5ºC (± 0.2). Seawater chemistry for the duration of the experiment is reported in Supplementary Material in Ericson *et al.* (2018b). Observational units (CO_2 treatment tanks) could not be replicated, due to the large tank size required to achieve the best possible animal husbandry for this pelagic species, and the limited space and resources available for these large tanks over such a long-term study. Tanks were inspected daily, and there was no visual evidence to suggest that tank effects were confounding our experimental results.

Two hundred krill were randomly assigned to each tank on the first day of the experiment (25th January 2016), and reared in these pCO_2 treatments until the experiment ended on the 12th December 2016. Light was controlled in the laboratory to mimic the seasonal Southern Ocean light regime (66°S, 30m depth) and krill were fed six days per week with a microalgal diet of the Antarctic species *Pyramimonas gelidicola* (2 x 10^4 cells mL^{-1}), and Reed Mariculture Inc. (USA) cultures of *Thalassiosira weissflogii* (8.8 x 10^3 cells mL^{-1}), *Pavlova lutheri* (4.5 x 10^4 cells mL^{-1}) and *Isochryisis galbana* (5.5 x 10 cells mL^{-1}).

5.3.2. Sample collection and lipid extraction

Krill were sampled from the pCO_2 treatment tanks in experimental weeks 1, 2, 4 and 5 (summer), 26 (winter), and 39, 41 and 43 (spring). Five to ten krill were sampled from each

tank during each sampling week (only three krill were sampled from the 4000 µatm pCO_2 tank due to increased mortality in that tank (see Ericson *et al.* 2018b) and lower overall numbers of krill). Individual krill were placed in cryo-tubes and frozen immediately at –80°C until needed for lipid analysis.

Krill were weighed (wet mass), and the length of each specimen was measured from the tip of the rostrum to the tip of the uropod using measurement 'Standard Length 1' (Kirkwood 1984). To prevent sample degradation, krill were kept frozen during the measuring process. A dry mass (g) for each krill sample was obtained by multiplying the wet mass by 0.2278 to account for the 77.2% water content in krill (Virtue *et al.* 1993a).

Krill specimens were added to separatory funnels and extracted using a modified Bligh and Dyer (1959) method consisting of a methanol:dichloromethane:water (MeOH:CH_2Cl_2:H_2O) solvent mixture (20:10:7 mL), and overnight extraction. Phase separation was carried out the following day by adding 10 mL CH_2Cl_2 and 10 mL saline MilliQ H_2O to each separatory funnel, giving a final MeOH:CH_2Cl_2:H_2O solvent ratio of 1:1:0.85. The lower layer was drained into a round bottomed flask, and the total solvent extract was concentrated using rotary evaporation. The concentrated extract was transferred into a pre-weighed 2 mL vial and the solvent was blown down under nitrogen (N_2) gas to obtain a total lipid extract (TLE) weight. Solvent (CH_2Cl_2) was added until further procedures were carried out to avoid oxidation.

5.3.3. Lipid class analysis

TLE were used to obtain the lipid class composition of each sample. Aliquots (1µl) of each TLE were spotted on chromarods and developed in a solvent bath of hexane:diethyl-ether:acetic acid (90:10:0.1 mL, v:v:v) for 25 min, before drying in an oven at 50ºC for 10 min.

Chromarods were placed in an Iatroscan MK-5 TLC/FID analyser (Iatron Laboratories, Tokyo, Japan) for analysis. A standard solution of known quantities of wax esters (WE), triacylglycerols (TAG), free fatty acids (FFA), sterols (ST), and phospholipids (PL) was used to confirm peak identities and to calibrate the flame ionisation detector. Lipid class peaks were labelled using SIC-480II Iatroscan Integrating Software v.7.0-E, quantified using predetermined linear regressions, and expressed as mg per g of krill dry mass (mg g DM $^{-1}$). Triacylglycerol data is presented in Ericson *et al.* (2018b). Only the PL to ST ratio is presented in this manuscript as we were primarily interested in investigating homeoviscous adaptation in krill.

5.3.4. Fatty acid analysis

To prepare fatty acid methyl esters (FAME), a subsample of the TLE was transferred to a glass test tube fitted with a Teflon lined screw cap, and treated with 3 mL methylating solution (MeOH : CH_2Cl_2 : HCl (hydrochloric acid), 10:1:1, v:v:v). The sample was then heated at 90 – 100°C for 1 hr 15 mins. Samples were cooled and 1 mL of H_2O and 1.8 mL of C_6H_{14} (hexane): CH_2Cl_2 solution was added to extract the FAME. Samples were then centrifuged for five minutes and the upper layer containing FAME was transferred to a vial. An additional 1.8 mL of C_6H_{14}:CH_2Cl_2 was added to the test tube and samples were centrifuged again. This process was repeated three times in total, and samples were blown down using N_2 gas in between transfers. FAME samples were made up to 1.5 mL with CH_2Cl_2 and stored at –20°C until further analysis. Prior to analysis, samples were blown down again using N_2 gas and 1.5 mL of internal injection standard (23:0 FAME) was added to each vial.

Samples were analysed via gas chromatography (GC-FID) using an Agilent Technologies 7890A GC System (Palo Alto, California USA) equipped with a non-polar

Equity™-1 fused silica capillary column (15 m x 0.1 mm internal diameter and 0.1 µm film thickness). Samples (0.2 µl) were injected in splitless mode at an oven temperature of 120°C with helium as the carrier gas. The oven temperature was raised to 270°C at a rate of 10°C per minute, then to 310°C at 5°C per minute. Agilent Technologies ChemStation software was used to quantify fatty acid peaks, with initial identification based on comparison of retention times with known (Nu Chek Prep mix) and laboratory (fully characterised tuna oil) standards. Fatty acid peaks were expressed as a percentage of the total fatty acid area.

Confirmation of component identification was performed by gas chromatography-mass spectrometry (GC-MS) of selected samples and was carried out on a Thermo Scientific 1310 GC coupled with a TSQ triple quadruple. Samples were injected using a Tripleplus RSH auto sampler using a non polar HP-5 Ultra 2 bonded-phase column (50 m x 0.32 mm i.d. x 0.17 µm film thickness). The HP-5 column was of similar polarity to the column used for GC analyses. The initial oven temperature of 45°C was held for 1 min, followed by an increase in temperature of 30°C per minute to 140°C, then at 3°C per minute to 310°C, where it was held for 12 minutes. Helium (He) was used as the carrier gas. The operating conditions of the GC-MS were: electron impact energy 70 eV; emission current 250 µamp, transfer line 310°C; source temperature 240°C; scan rate 0.8 scan/sec and mass range 40 - 650 Da. Thermo Scientific XcaliburTM software (Waltham, MA, USA) was used to process and acquire mass spectra.

Mean fatty acid chain length (MCL) was calculated using the equation from Bennett *et al.* (2018):

$$MCL = \Sigma \text{ (mg fatty acid g lipid}^{-1} \text{ x C) / total mg fatty acid g lipid}^{-1}$$

where C = number of carbon atoms

5.3.5. Statistical analyses

Principal component analyses (PCA) were carried out in PRIMER 6 (http://www.primer-e.com). Pearson correlation was used due to differences in fatty acid variances, and data were transformed (log x+1) before analysis. All other statistical analyses were carried out in RStudio (v 1.1.453; www.rstudio.com). Total lipid, specific fatty acids, lipid class and fatty acid ratios were analysed using Two Way ANOVA with pCO_2 and week as main effects, and a pCO_2*week interaction term. Tukey comparisons were used to compare levels of pCO_2 with one another. On visual assessment of the data, weeks 1 – 5 were analysed as a group, and weeks 26 – 43 were analysed as a separate group, as the groups had heterogeneous variances and represented two distinct data sets. Type 3 Sums of Squares were used as the sampling regime was unbalanced. Log or square root transformations were applied when assumptions of normality and/or homogeneity of variances were not met. For Two Way ANOVA of total lipid data, one outlier was removed from the statistical analysis in order to meet assumptions of homogeneity of variances. Principal component figures were created in PRIMER 6, and all other figures were created using the RStudio packages ggplot2, plyr and dplyr.

5.4. Results

5.4.1. Effect of pCO_2 on total lipid and phospholipid/sterol ratios in krill

Quantities of total lipid in krill in weeks 1 – 5 did not differ between pCO_2 treatments or weeks (pCO_2; $p = 0.577$, week; $p = 0.097$; pCO_2*week; $p = 0.165$). During these first five weeks of the experiment, average quantities of total lipid in krill (Figure 5.1A) were 57.4 ± 19.8 mg g^{-1} dry mass (DM; mean ± SD). During weeks 26 – 43 (Figure 5.1A), there was a fourfold increase in average total lipid in krill to 273.8 ± 75.4 mg g^{-1} DM (mean ± SD), and the effect of pCO_2 on total lipid differed between weeks (Two Way ANOVA; pCO_2*week, $p = 0.052$).

Krill in 4000 µatm pCO_2 seawater had lower quantities of total lipid than krill in all other pCO_2 treatments during week 26 (Tukey $p < 0.003$). They also had lower total lipid than krill in 400 and 2000 µatm pCO_2 during week 41 (Tukey $p < 0.046$). During weeks 39 and 43, the quantities of total lipid in krill did not differ between pCO_2 treatments ($p > 0.930$).

Ratios of PL/ST in krill (Figure 5.1B) did not differ between pCO_2 treatments during weeks 1 – 5 (Two Way ANOVA pCO_2*week, $p = 0.533$). Krill in 4000 µatm pCO_2 seawater had higher average PL/ST ratios than krill in 400 µatm pCO_2 seawater during weeks 26 – 43, but these were significantly higher only in weeks 39 (Tukey $p < 0.001$) and 41 (Tukey $p < 0.001$).

5.4.2. Principal component analysis of fatty acid percentage composition

Fifty-eight fatty acids were found in krill. Only fatty acids at percentages of ≥ 0.5% of total fatty acids (17 fatty acids) are analysed and presented in the following results.

Fatty acid percentage data for adult krill collected during weeks 1, 2, 4 and 5 (summer) were similar, and data collected during weeks 26 – 43 (winter and spring) were similar, so data were combined into these two separate groups for principal component analysis (PCA). Results of PCA analyses for individual weeks can be found in Appendix XII.

Fatty acid percentage composition of krill did not differ between pCO_2 treatments during weeks 1 – 5 when analysed using PCA (Figure 5.2A). Principal component 1 (PC1) separated krill with higher percentages of LC-PUFA from those with higher percentages of 14:0 and medium-chain (C_{16} – C_{18}) monounsaturated fatty acids (MUFA) and PUFA, but no separation of pCO_2 treatments was observed along PC1(x-axis) or principal component 2 (PC2; y-axis).

Krill fatty acid percentage composition differed between pCO_2 treatments during weeks 26 – 43 (Figure 5.2B). PC1 clearly separated krill in 4000 µatm pCO_2 seawater from krill in

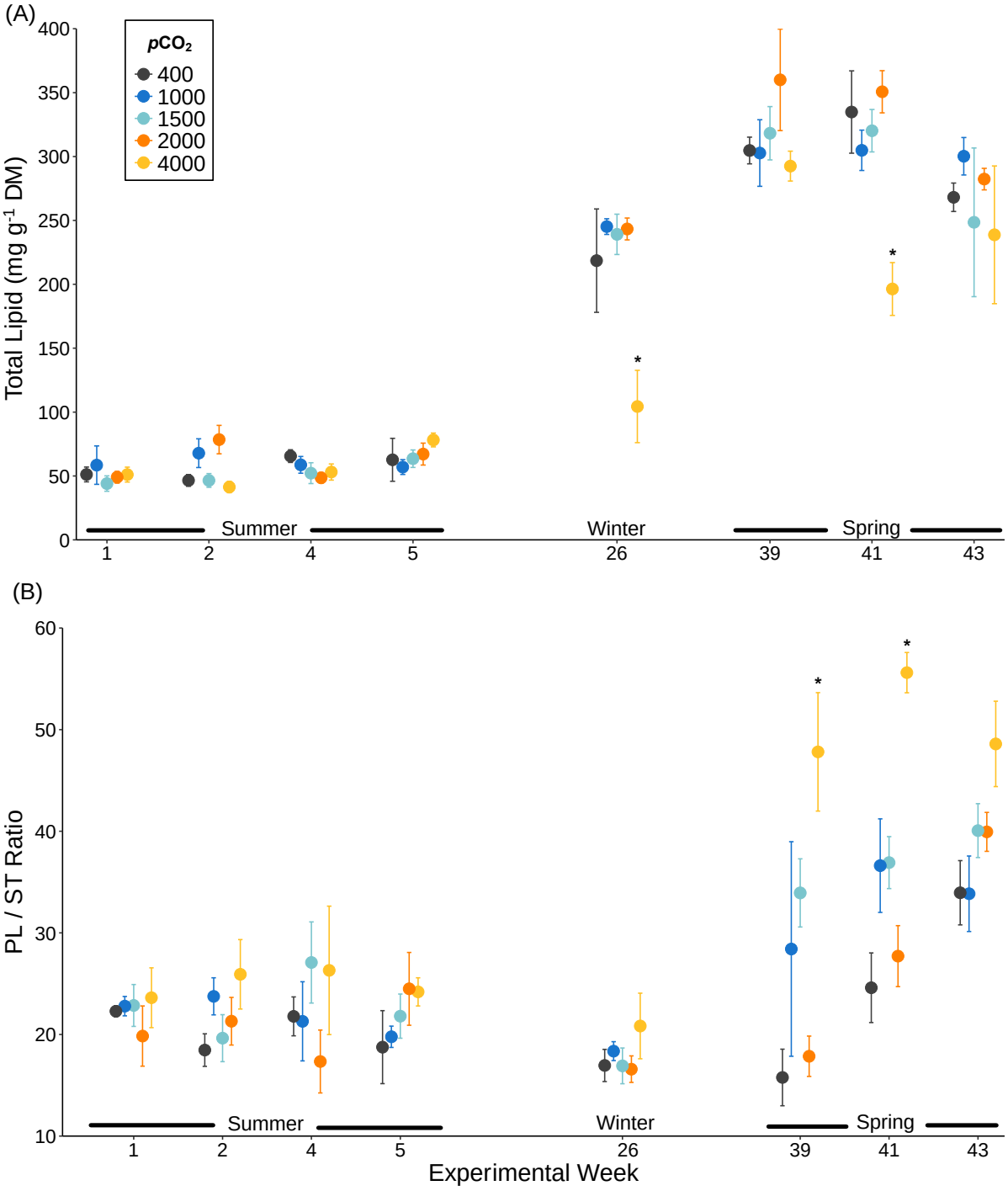


Figure 5.1. (A) Total lipid (mg g $^{-1}$ dry mass; mean ± SE) and **(B)** phospholipid/sterol ratio (mean ± SE) of *Euphausia superba* in weeks 1, 2, 4, 5, 26, 39, 41 and 43 of the one-year ocean acidification experiment. For each pCO_2 treatment and week $n = 3 – 7$. Statistically significant differences to ambient seawater (400 µatm pCO_2) are highlighted with an asterisk ($p < 0.05$).

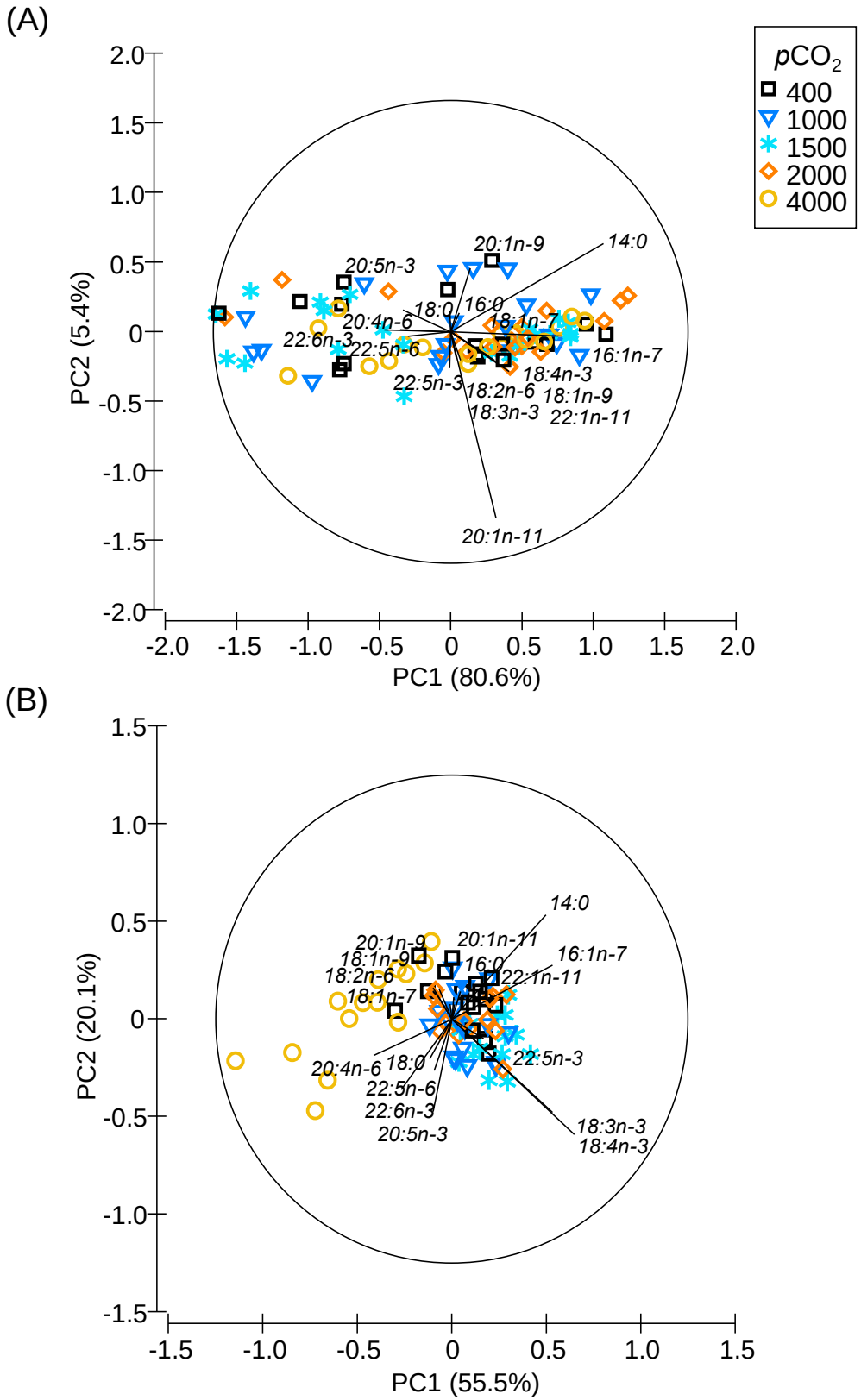


Figure 5.2. Principal component analyses of the fatty acid percentage composition of *Euphausia superba* in 400, 1000, 1500, 2000 and 4000 µatm pCO_2 during **(A)** weeks 1 – 5 (summer) and **(B)** weeks 26 – 43 (winter and spring) of the one-year ocean acidification experiment. The amount of variation (%) explained by each principal component (PC) is shown on the x-axis (PC1) and y-axis (PC2).

other pCO_2 treatments (Figure 5.2B). Krill in 4000 µatm pCO_2 seawater had higher percentages of LC-PUFA 20:4n-6 and 22:6n-3 than krill in 400 – 2000 µatm pCO_2 seawater. Krill in 400 – 2000 µatm pCO_2 treatments had higher percentages of 14:0, 16:1n-7, 18:3n-3 and 18:4n-3 (Figure 5.2B). PC2 separated krill with higher percentages of 14:0 and 16:1n-7 (predominantly krill in 400 µatm pCO_2) from those with higher percentages of 18:3n-3 and 18:4n-3 (predominantly krill in 1500 µatm pCO_2) and those with higher percentages of 18:2n6, 18:1n9 and 20:5n-3 and 22:6n-3 (predominantly krill in 4000 µatm pCO_2). Principal component loadings for each fatty acid included in the PCA analyses are shown in Appendix XIII.

Percentages (mean ± SD) of the eight fatty acids with the highest PCA loadings (14:0, 16:1n-7c, 18:3n-3, 18:4n-3, 18:2n-6, 20:4n-6, 20:5n-3, 22:6n-3) in each pCO_2 treatment are shown in Appendix XIV (weeks 1 – 5; no significant differences, $p > 0.05$) and Table 5.1. (weeks 26 – 43).

5.4.3. Fatty acid indicators of homeoviscous adaptation and immune responses in krill

Seawater pCO_2 did not affect the MCL, PUFA/SFA ratio, 22:6n-3/20:4n-6 ratio and 18:3n-3/18:2n-6 ratio in krill during weeks 1 – 5 (Appendix XV), but these ratios were altered during weeks 26 – 43 (Figure 5.3). MCL was higher in krill in 4000 µatm pCO_2 than krill in other treatments in week 26 (Two Way ANOVA pCO_2*week $p = 0.049$, Tukey $p < 0.05$). The PUFA/SFA ratio did not differ between krill in different pCO_2 treatments (Two Way ANOVA pCO_2*week, $p = 0.089$; pCO_2, $p = 0.101$). Krill in 4000 µatm pCO_2 had a lower 22:6n-3/20:4n-6 ratio than krill in other treatments during weeks 26, 39, 41 and 43 (Two Way ANOVA pCO_2*week = 0.571; pCO_2, Tukey $p < 0.001$). Krill in 1500 µatm pCO_2 had higher 18:3n-3/18:2n-6 ratios than krill in other treatments (Two Way ANOVA pCO_2*week $p = 0.100$; pCO_2 Tukey $p < 0.001$) and krill in 4000 µatm pCO_2 had lower 18:3n-3/18:2n-6 ratios than krill in

other treatments during all weeks from 26 – 43 (ANOVA pCO_2*week, $p = 0.100$; pCO_2, Tukey $p < 0.05$).

Table 5.1. Percentage composition (mean ± SD) of selected fatty acids in *Euphausia superba* reared in 400, 1000, 1500, 2000 and 4000 pCO_2 seawater during experimental weeks 26, 39, 41 and 43 of the one-year ocean acidification experiment. Values in bold type are those that are significantly different (Two way ANOVA pCO_2*week and Tukey comparisons; $p < 0.05$) to ambient seawater (400 µatm pCO_2). Red arrows illustrate whether there are lower levels (↓) or higher levels (↑) of the fatty acid in krill compared with those in ambient seawater. For each pCO_2 treatment $n = 3 – 5$.

Fatty Acid	pCO_2	Week 26	Week 39	Week 41	Week 43
14:0	400	4.55 ± 0.59	4.96 ± 0.34	5.57 ± 0.51	5.10 ± 0.37
	1000	4.54 ± 0.40	4.92 ± 0.70	4.38 ± 0.43	4.88 ± 0.44
	1500	4.50 ± 0.76	5.05 ± 0.31	4.08 ± 1.13	4.90 ± 0.26
	2000	4.53 ± 0.28	4.92 ± 0.45	4.63 ± 0.68	4.85 ± 0.37
	4000	**2.98 ± 0.97** ↓	4.97 ± 0.38	**3.47 ± 0.15** ↓	4.17 ± 0.45
16:1n-7c	400	5.55 ± 0.34	6.04 ± 0.27	6.50 ± 0.56	5.90 ± 0.47
	1000	5.42 ± 0.18	5.78 ± 0.56	5.66 ± 0.57	5.84 ± 0.38
	1500	5.83 ± 0.64	6.08 ± 0.43	5.83 ± 0.50	5.50 ± 0.53
	2000	5.30 ± 0.14	5.85 ± 0.65	5.77 ± 0.55	5.65 ± 0.44
	4000	**3.72 ± 1.06** ↓	**4.77 ± 0.55** ↓	**4.27 ± 0.06** ↓	**4.43 ± 0.47** ↓
18:2n-6	400	8.80 ± 0.36	9.10 ± 0.24	9.13 ± 0.78	9.56 ± 0.47
	1000	8.78 ± 0.29	9.12 ± 0.13	9.18 ± 0.25	9.30 ± 0.25
	1500	**8.28 ± 0.17** ↓	**8.70 ± 0.14** ↓	**8.85 ± 0.47** ↓	**8.93 ± 0.21** ↓
	2000	8.90 ± 0.18	9.30 ± 0.33	9.43 ± 0.59	9.18 ± 0.13
	4000	**8.90 ± 0.48** ↑	**10.07 ± 0.25** ↑	**10.30 ± 0.10** ↑	**9.80 ± 0.80** ↑
18:3n-3	400	3.10 ± 0.26	3.76 ± 0.50	3.67 ± 0.45	3.36 ± 0.51
	1000	3.80 ± 0.60	3.80 ± 0.50	3.84 ± 0.30	3.70 ± 0.20
	1500	**4.20 ± 0.22** ↑	**4.08 ± 0.26** ↑	**4.58 ± 0.36** ↑	**4.10 ± 0.44** ↑
	2000	3.40 ± 0.18	3.77 ± 0.17	3.87 ± 0.25	4.10 ± 0.35
	4000	**2.68 ± 0.34** ↓	**2.67 ± 0.23** ↓	**2.40 ± 0.20** ↓	**2.67 ± 0.15** ↓
18:4n-3	400	0.62 ± 0.13	1.04 ± 0.27	0.93 ± 0.06	0.82 ± 0.19
	1000	0.84 ± 0.27	1.02 ± 0.29	1.08 ± 0.18	0.90 ± 0.14
	1500	**1.18 ± 0.22**	**1.12 ± 0.15**	**1.35 ± 0.25**	**1.27 ± 0.35**
	2000	0.72 ± 0.10	1.00 ± 0.14	0.93 ± 0.06	1.15 ± 0.26
	4000	**0.40 ± 0.25** ↓	**0.50 ± 0.00** ↓	**0.47 ± 0.06** ↓	**0.60 ± 0.10** ↓
20:4n-6	400	1.60 ± 0.24	1.52 ± 0.16	1.40 ± 0.10	1.42 ± 0.04
	1000	1.42 ± 0.18	1.52 ± 0.19	1.58 ± 0.13	1.50 ± 0.07
	1500	1.52 ± 0.10	1.32 ± 0.10	1.45 ± 0.17	1.30 ± 0.10
	2000	1.62 ± 0.10	1.40 ± 0.08	1.47 ± 0.21	1.38 ± 0.10
	4000	**2.38 ± 0.45** ↑	1.70 ± 0.10	**2.03 ± 0.31** ↑	1.63 ± 0.06
20:5n-3	400	6.08 ± 0.39	6.78 ± 0.80	6.90 ± 0.30	6.32 ± 0.56
	1000	6.20 ± 0.43	7.03 ± 0.79	7.54 ± 0.38	7.18 ± 0.49
	1500	7.12 ± 0.45	7.00 ± 0.22	7.62 ± 1.15	6.87 ± 0.25
	2000	6.58 ± 0.31	6.62 ± 0.42	6.70 ± 0.36	7.08 ± 0.33
	4000	**7.96 ± 1.15** ↑	6.53 ± 0.31	7.13 ± 0.47	7.07 ± 0.81
22:6n-3	400	10.62 ± 1.07	10.84 ± 0.36	9.70 ± 0.85	10.70 ± 0.54
	1000	10.42 ± 0.33	10.60 ± 0.47	11.18 ± 0.99	10.82 ± 0.41
	1500	10.57 ± 1.10	10.45 ± 0.37	10.78 ± 1.15	11.20 ± 0.72
	2000	10.55 ± 0.62	10.38 ± 1.13	10.70 ± 0.46	10.57 ± 0.41
	4000	**13.38 ± 2.38** ↑	10.23 ± 1.46	12.07 ± 0.76	10.70 ± 0.62

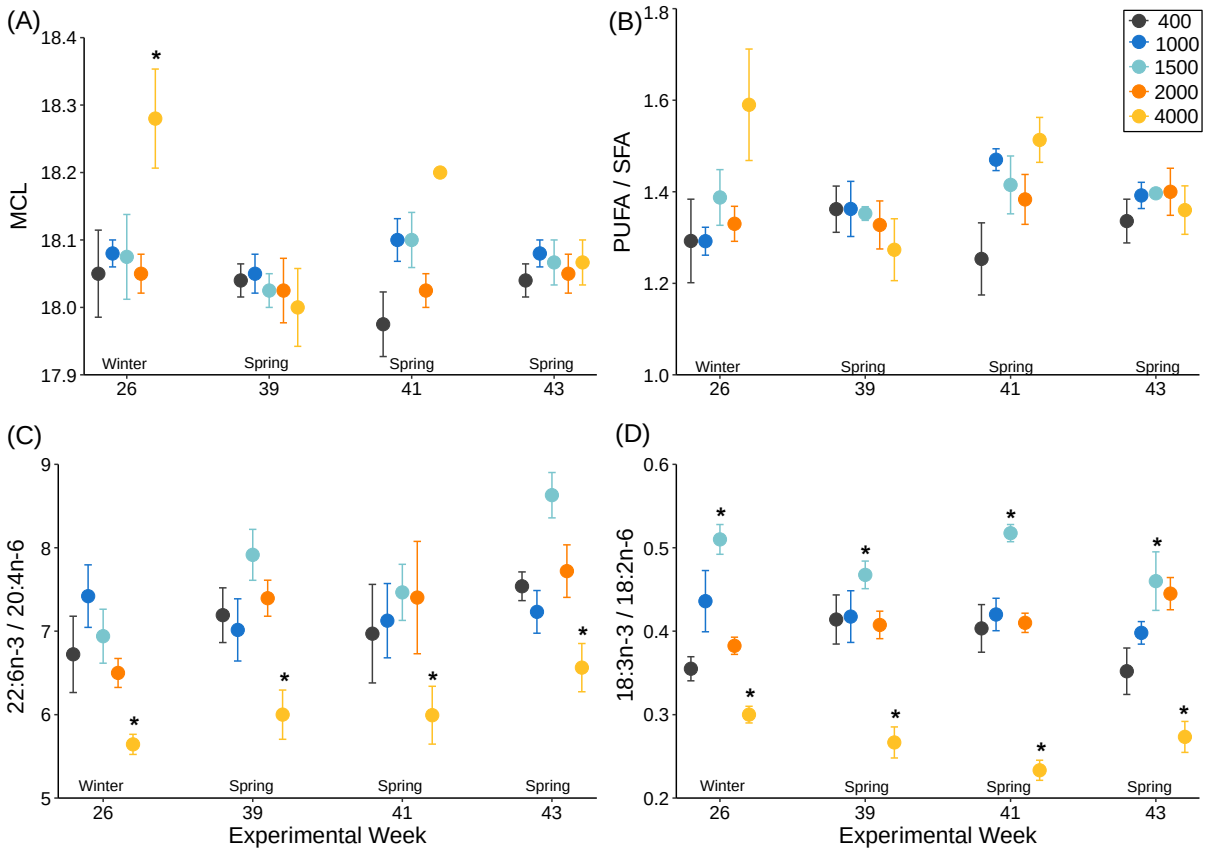


Figure 5.3. Fatty acid indicators of homeoviscous adaptation and immune responses in *Euphausia superba* exposed to 400, 1000, 1500, 2000 and 4000 µatm pCO_2 in experimental weeks 26, 39, 41 and 43, where (**A**) Mean chain length (MCL); (**B**) ratio of polyunsaturated to saturated fatty acids (PUFA/SFA); (**C**) ratios of docosahexaenoic acid/arachidonic acid (22:6n-3/20:4n-6); (**D**) alpha-linolenic acid/linoleic acid (18:3n-3/18:2n-6). All values are mean ± SE. Statistically significant differences to ambient seawater (400 µatm pCO_2) are highlighted with an asterisk ($p < 0.05$).

5.5. Discussion

Krill reared in ambient seawater pCO_2 levels (400 µatm pCO_2), and those levels predicted for the near-future (100 – 300 years; 1000 – 2000 µatm pCO_2) did not have significantly different quantities of total lipid (mg g $^{-1}$ DM) or ratios of PL/ST. We observed no effects of near-future pCO_2 on fatty acid composition during weeks 1 – 5 of the experiment (summer). In winter and spring (weeks 26 – 43), elevated percentages of C_{18} fatty acids 18:4n-3 and 18:3n-3 were measured in krill in 400 – 2000 µatm pCO_2 and were highest in krill in 1500 µatm pCO_2. Krill in extreme pCO_2 (4000 µatm), had a different lipid composition to those in 400 – 2000 µatm

pCO_2 treatments during winter and spring. Some krill had lower quantities of total lipid, higher PL/ST ratios and MCL, and all krill had consistently lower ratios of n-3/n-6 fatty acids (22:6n-3/20:4n-6 and 18:3n-3/18:2n-6).

The absence of any *p*CO_2 effect on krill biochemistry during the first five weeks of the experiment suggests that it may take some time before changes can be detected in the lipid profile of adult krill. Numerous short-term ocean acidification studies have, however, detected changes in the lipid and fatty acid profile of other organisms, over time periods substantially shorter than or equal to five weeks (Rossoll *et al.* 2012; Wynn-Edwards *et al.* 2014; Díaz-Gil *et al.* 2015; Valles-Regino *et al.* 2015; Wang *et al.* 2017; Gao *et al.* 2018). Krill have higher metabolic rates during summer (Meyer & Teschke 2016). A higher metabolic rate may enable krill to more efficiently regulate acid-base balance and other vital functions such as their lipid biochemistry. This could explain why effects of extreme *p*CO_2 on krill biochemistry were not observed during summer, and only evident in winter and mid-spring when metabolic rates are lower. The interaction between seasonal metabolic rates, lipid biochemistry and increased *p*CO_2 at different time scales is a topic for further study.

5.5.1. Effects of near-future pCO₂ on krill lipids

Many ocean acidification studies to date have found no effect of near-future *p*CO_2 on total lipid levels in organisms (Schram *et al.* 2011; Matson *et al.* 2012; Carter *et al.* 2013; Rivest & Hofmann 2015; Valles-Regino *et al.* 2015; Strahl *et al.* 2016; Ab Lah *et al.* 2018; Bennett *et al.* 2018). Lipids are an important energy source and essential for physiological function and survival, therefore, organisms are likely to maintain relative lipid levels unless they are under substantial physiological stress.

Like other laboratory studies (Brown 2010; Höring *et al.* 2018), krill in our study displayed seasonal fluctuations in lipid mass even when given a constant food supply. This occurred in all pCO_2 treatments, indicating that endogenous rhythms entrained by the seasonal light cycle were the dominant driver controlling lipid deposition in krill (Höring *et al.* 2018).

Our finding that near-future pCO_2 did not have a significant effect on total lipid mass in adult krill suggests that ocean acidification does not affect their ability to feed or store fat. This corresponds well with Ericson *et al.* (2018b), which indicates that physiological processes in adult krill are unaffected by near-future acidification. Animals may preferentially retain lipids and utilize protein as an energy source when exposed to near-future pCO_2 (Carter *et al.* 2013), or maintain lipid and protein levels but grow at a slower rate (Strahl *et al.* 2016). Adult krill in near-future pCO_2, however, do not display slow or delayed growth compared with those in ambient seawater (see Ericson *et al.* 2018b). A previous study found that krill exposed to 750 µatm pCO_2 for 24 hours had slightly lower protein content than krill in ambient pCO_2 seawater (Saba *et al.* 2012), suggesting that they may switch from lipid to protein catabolism in high pCO_2 conditions.

Near-future pCO_2 did not significantly alter the composition of fatty acids associated with immune function (n-3/n-6 ratios) and cell membrane fluidity (MCL, PUFA/SFA and PL/ST) in krill. This is a further indication that these levels of pCO_2 do not induce physiological stress. Cell membrane fatty acid composition is tightly regulated by temperature (Los & Murata 2004) and may be driven more by the cold temperatures krill are adapted to (Corsolini & Borghesi 2017). Ambient seawater temperatures (0.5°C) were used in this study, which could explain the stability of these fatty acid ratios. Elevated seawater temperatures may influence fatty acid composition more than acidification, although previous studies indicate that krill lipids are not altered by temperatures up to 4°C above ambient (Brown 2010).

5.5.2. Effects of extreme pCO_2 on krill lipids

Decreases in total lipid and increases in levels of inflammatory n-6 PUFA in krill reared in 4000 µatm pCO_2, suggest that unlike krill in 400 – 2000 µatm pCO_2, these krill were physiologically stressed. Lipid depletion observed during selected weeks in winter and spring corresponds with decreases in quantities of storage lipid (triacylglycerol) in these krill (see Ericson *et al.* 2018b). Physiological processes such as growth and maturation, along with acid-base regulation required in extreme seawater pCO_2, are energetically expensive (Whiteley 2011) and these processes may have depleted lipid reserves. Feeding in these krill may have also been compromised and caused a decrease in total lipid, although feeding rates were not measured in this study.

Krill in 4000 µatm pCO_2 seawater may have been storing 20:4n-6 for production of inflammatory eicosanoids, and for ion transport (Stanley-Samuelson 1987; Van Anholt 2004; Rowley *et al.* 2005), in an attempt to regulate immune responses and maintain intra- and extra-cellular pH in this extreme environment. Such increases in n-6 fatty acids have been observed in fish (Murray *et al.* 2017) and shrimp (Gao *et al.* 2018) exposed to acidification. Inflammation is important for organism health and tissue repair, but excessive inflammation is maladaptive (Calder 2010).

As levels of n-6 fatty acids in organisms increase, levels of n-3 fatty acids decrease, as the elongation-desaturation pathways for n-3 and n-6 fatty acids compete for the same enzymes (Monroig & Kabeya 2018). The lower n-3/n-6 ratios in krill in 4000 µatm pCO_2 during winter and spring may, therefore, correspond to a shift in elongation-desaturation pathways used by these krill. The increase in n-3 PUFA in krill up to 1500 µatm pCO_2, followed by a decrease down to 4000 µatm pCO_2, suggests that 1500 µatm pCO_2 may be the point at which krill fatty

acid composition switches from an anti-inflammatory status (more n-3 PUFA) to a pro-inflammatory status (more n-6 PUFA).

Cell membrane alteration via homeoviscous adaptation has been most commonly explored with respect to changing temperatures (Hazel 1995; Pörtner *et al.* 2007; Hixson & Arts 2016), but other factors such as salinity, hypoxia (Ernst *et al.* 2016) and seawater pH (Bennett *et al.* 2018) can alter membrane structure. The higher ratios of PL/ST in krill in 4000 µatm pCO_2 in winter and spring suggests that krill may have been actively increasing membrane fluidity, to enable more efficient exchange of ions across their cell membranes and control acid-base balance. Alternatively, the ability of krill in 4000 µatm pCO_2 to maintain an optimal ST composition may have been compromised. This could lead to membrane 'hyper-fluidity' and disrupt cellular function (Bennett *et al.* 2018). Under hypercapnic stress, homeoviscous adaptation through regulation of lipid class ratios (e.g. PL/ST) may be more energy efficient than modification of PUFA/SFA ratios and MCL, which remained relatively stable in krill in 4000 µatm pCO_2.

5.5.3. Laboratory effects and other climate change stressors

The fatty acid profile of krill in our laboratory study also reflects their aquarium diet and does differ to that of wild krill. Ratios of 22:6n-3/20:4n-6, 20:5n-3/22:6n-3, and 18:3n-3/18:2n-6 are higher in wild krill (Virtue *et al.* 1996; Hagen *et al.* 2001; Ericson *et al.* 2018a) than were observed for our laboratory reared krill, indicating that wild krill have higher n-3/n-6 ratios. The diet of wild krill is not replicable in the laboratory (Brown *et al.* 2010), but the higher n-3/n-6 ratios of these krill may influence and even further enhance their resilience to elevated pCO_2, due to their higher levels of anti-inflammatory fatty acids. Levels of krill prey in the Southern Ocean also fluctuate spatially and temporally (Schmidt & Atkinson 2016), and krill

in our study were fed a constant supply of food. Krill increase their feeding rates when exposed to high pCO_2 (Saba *et al.* 2012), possibly to maintain enough energy for physiological processes under pCO_2 stress (Saba *et al.* 2012, Ericson *et al.* 2018b). Changing food levels both seasonally, regionally and with climate change may, therefore, also influence how wild krill respond to ocean acidification.

Krill will be exposed to multiple climate change stressors in the future, in addition to ocean acidification (Rintoul *et al.* 2018). Rapid warming is already evident in the West Antarctic Peninsula region (Vaughan *et al.* 2003), both at the sea surface (Meredith & King 2005), and in the deep ocean (Antarctic Bottom Water (Schmidtko *et al.* 2014)). In laboratory studies, simulated ocean warming significantly affects the fatty acid composition of some organisms (Garzke *et al.* 2016; Feijão *et al.* 2017; Bennett *et al.* 2018; Bi *et al.* 2018; Hernando *et al.* 2018; Malekar *et al.* 2018). A previous long-term laboratory study found only minor differences between the lipid and fatty acid composition of krill reared in -1°C, 1°C and 3°C (Brown 2010). The temperature range used in the Brown (2010) study was within the range that krill experience in their natural environment (krill are abundant at South Georgia where seawater temperatures reach 5°C (Schmidt & Atkinson 2016)), therefore, the temperatures may not have been high enough to detect significant temperature effects. Further studies are needed to establish whether the combined effects of increased seawater temperature and pCO_2 affect the lipid and fatty acid composition of krill.

5.6. Conclusions

Lipid mass and fatty acid composition in adult krill were unaffected when krill were exposed to near-future levels of pCO_2 (1000 – 2000 µatm) in the laboratory. Extreme pCO_2 altered the lipid and fatty acid content and composition of krill, although consistent differences were not

observed across all experimental weeks. Extreme pCO_2 had no effect on krill lipid biochemistry during summer, but during selected weeks in winter and spring, krill in 4000 µatm pCO_2 had elevated levels of inflammatory omega-6 fatty acids and showed evidence of increased membrane fluidity. These observations suggest that krill may be less able to tolerate elevated pCO_2 conditions during winter and spring, when metabolic rates are lower and reproductive maturation occurs. Seawater pH levels are also lower in the Antarctic in the winter than summer (McNeil & Matear 2008), and prey availability is lower in winter in some areas of the Southern Ocean (Schmidt & Atkinson 2016). Collectively, these factors may influence how krill respond to near-future pCO_2 in the wild, and determine their resilience in a future high CO_2 world.

5.7. Acknowledgements

We thank Tasha Waller, Ashley Cooper, Blair Smith and Rob King for their invaluable technical assistance in the krill aquarium, and Guang Yang for assistance with daily experimental monitoring. This research was funded by an Australian Research Council Linkage Grant LP140100412 between the Australian Antarctic Division, Commonwealth Scientific and Industrial Research Organisation, Institute for Marine and Antarctic Studies (University of Tasmania), Aker Biomarine and Griffith University.

6 General Discussion

6.1. Importance of krill and the current status of krill populations

The importance of Antarctic krill (*Euphausia superba*, hereafter krill) in the Southern Ocean ecosystem has been extensively reviewed (Marr 1962; Siegel 2016a; Nicol 2018). Their role as nutritious prey for higher trophic levels (Trathan & Hill 2016), and as important recyclers of iron (Ratnarajah *et al.* 2018) makes them a keystone species in the Antarctic. Substantial declines in krill biomass in the Southern Ocean could cause a major ecological regime shift (Loeb *et al.* 1997; Atkinson *et al.* 2004; Ward *et al.* 2018). The resilience and survival of krill populations is, therefore, vital for the whole Southern Ocean ecosystem.

An earlier study indicated that krill populations declined by up to 80% between the 1970s and 2003 (Atkinson *et al.* 2004). More recent studies have found that krill biomass is significantly higher than previous estimates (Atkinson *et al.* 2009), and that populations appear stable despite interannual variations in biomass (Cox *et al.* 2018). These recent studies suggest that krill populations are currently healthy, but substantial anthropogenic activities in the Southern Ocean during the 20th Century and into the 21st Century need to be considered, in order to predict the health of krill populations into the future.

Intensive commercial whaling was undertaken in Antarctica during the 20th Century (Aronson *et al.* 2011); more than two million whales were killed and many species were hunted to near-extinction (Claphan *et al.* 1999). Commercial whaling in the Southern Ocean ceased in 1987 (Trathan & Reid 2009), and some baleen whale species are showing signs of recovery (Branch *et al.* 2006; Nicol 2018). Predation pressure on krill by whales is likely to increase as whale populations recover (Trathan & Hill 2016; Nicol 2018). The fishery for krill has also expanded in recent years. Annual catch levels have regularly exceeded 200,000 tonnes during the years 2010 – 2017 (CCAMLR 2017); and the last time this occurred was during the krill

fishery boom between 1979 – 1992 (CCAMLR 2017). Although the krill fishery is strictly regulated and managed using a precautionary approach (Nicol *et al.* 2012), any substantial increase in predation by top predators (including humans) may impact krill biomass. In the 21st Century and beyond, the Southern Ocean will experience increases in seawater temperature and ocean acidification, increases in biological invasions and human presence, and decreases in sea ice extent (Rintoul *et al.* 2018). The ability of krill to exploit their current and future environment will determine the status of krill populations into the future.

This thesis combined samples collected from krill fishing vessels with live animal laboratory experiments, to provide the first synthesis of the biochemistry and physiology of krill in the present day, and in a future high CO_2 world. The results of this work significantly furthered our understanding of (a) seasonal and interannual fluctuations in lipid class and fatty acid composition, which allowed a detailed insight into krill diet, and (b) the resilience of krill physiology and biochemistry to future ocean acidification. In the following sections, I summarise the findings of this thesis, and explore the significance and future application of this research.

6.2. Krill biochemistry in the present day

In Chapter 2 it was demonstrated through the use of fatty acid dietary biomarkers, that krill diet changes seasonally, interannually and regionally. The fatty acid data indicated that krill consume phytoplankton (16:1n-7c, 18:4n-3, 20:5n-3, 22:6n-3 indicators), marine snow ($\sum C_{15} + C_{17} + C_{19}$ isomers), and other zooplankton (enhanced 18:1n-9c / 18:1n-7c ratio) including copepods (Σ 20:1 + 22:1 isomers). There were only minor differences in levels of fatty acid biomarkers between male and female krill, and these differences were not consistent between seasons or years. Krill displayed year-round omnivory, but the level of omnivory increased as the seasons progressed from summer to early spring. Quantities of essential omega-3 fatty acids

20:5n-3 and 22:6n-3 in krill (biomarkers for diatoms and dinoflagellates, respectively), showed clear seasonal fluctuations, with highest quantities present in autumn krill in all fishing years (2014, 2015 and 2016). Quantities of these fatty acids in krill were closely correlated with total lipid mass (Hellessey *et al.* 2018). Levels and quantities of the diatom marker 16:1n-7c and flagellate marker 18:4n-3 were more variable in krill, and did not show strong seasonal trends. Regional differences were also evident, but there was substantial interannual variability.

Detection of these spatial and temporal differences in the inferred diet of krill was possible due to the unprecedently large sample size obtained from the krill fishery. Through the use of fisheries-derived samples, this study filled significant knowledge gaps on the diet of krill, particularly for the winter months. This research used the most comprehensive sampling regime of any krill lipid study to date, and clearly demonstrates the utility of fisheries samples for Antarctic marine science. The fishery is currently concentrated in the Scotia Sea region, but the likely expansion into other regions of the Southern Ocean (as indicated by fishing notifications from China in recent CCAMLR reports (CCAMLR 2016, 2017)) may enable collection of fisheries samples on a larger spatial scale. Biochemical analyses of krill samples from all regions of the Southern Ocean during all seasons would be advantageous, but at the present time this is logistically unfeasible. The use of such samples for krill research is likely to become more mainstream and beneficial into the future (Kawaguchi & Nicol 2007; Schmidt & Atkinson 2016), and will significantly broaden the sampling scope for scientific research.

In Chapter 3, selected krill samples collected by the fishery in 2016 were analysed in further detail, to examine the fatty acid composition of separate structural (phospholipids) and storage lipids (triacylglycerols), and the sterol composition of male krill in different seasons. The essential omega 3 fatty acids 20:5n-3 and 22:6n-3 were strongly associated with the phospholipids, which further substantiates their important role in cell membranes.

Triacylglycerol fatty acid profiles (which are thought to best represent diet (Virtue *et al.* 1993a)) were used to infer that krill sampled in 2016 were consuming more flagellates in summer (higher levels of 18:4n-3), and more diatoms in autumn, winter and spring (higher levels of 16:1n-7). Krill were also more carnivorous in autumn (higher levels of ∑ 20:1 + 22:1 and higher 18:1n-9c/18:1n-7c ratios). These results corresponded well with those in Chapter 2 (which presented fatty acid data from the same whole krill samples collected in 2016). This finding challenges the paradigm that triacylglycerol fatty acids are more reliable than whole krill samples when making dietary inferences. Lipid class separation becomes unfeasible when large sample sizes are used, and results from Chapters 2 and 3 confirm that whole krill samples can be used for dietary biomarker studies, as long as the role of different lipid classes are considered when interpreting results (Stübing & Hagen 2003).

Approximately equal levels of 18:4n-3 were found in krill triacylglycerols and phospholipids during autumn, winter and spring, while levels of the copepod indicator ∑ 20:1 + 22:1 were generally higher in the phospholipids. This was an unexpected discovery, as 18:4n-3 (Clarke 1980; Falk-Petersen *et al.* 2000; Stübing *et al.* 2003) and ∑ 20:1 + 22:1 (Clarke 1980; Fricke *et al.* 1984) are usually highest in the triacylglycerol fraction in krill. Krill may mobilise 18:4n-3 to phospholipids for biosynthetic conversion to LC-PUFA, at times when LC-PUFA are less available in their diet. This could explain the decrease in the relative levels of 18:4n-3 in the triacylglycerols in autumn, winter and spring.

For the first time, it was shown that sterol composition varies with season, where the major krill sterol cholesterol was significantly higher in winter and spring. Cholesterol is likely the main driver of sterol composition in krill, and they may have been storing it for increased moulting and reproductive processes which occur in subsequent months.

It is clear from the above results, that krill are able to exploit their seasonally changing environment by employing a highly flexible diet. The omnivorous diet of many Antarctic invertebrates is likely to have developed due to the extreme seasonality of the Southern Ocean, and its large fluctuations in sea ice extent, solar radiation and primary production (Clarke 1988). Opportunistic omnivory is favourable in a heterogenous environment, as organisms can exploit a large range of food sources when they are encountered, and switch prey depending on availability (Coll & Guershon 2002; Kratina *et al.* 2012). It should be noted that not all prey types are nutritionally equal (Coll & Guershon 2002), and the predominantly herbivorous diet of wild krill is likely attributed to the high levels of nutritious essential omega-3 fatty acids in phytoplankton (Bottino 1974; Adarme-Vega *et al.* 2014). Previous feeding studies have shown that laboratory reared krill actively feed on copepods even when phytoplankton is readily available (Atkinson & Snyder 1997; Price *et al.* 1988). This flexible feeding strategy may be a major reason why krill are so successful (Bernard *et al.* 2018), and are one of the most abundant organisms on Earth (Tarling & Fielding 2016).

This study did not use fatty acid biomarkers to investigate the diet of larval and juvenile krill, as it was outside the scope of this thesis. The ability of these early life stages to access prey and store lipid will ultimately determine recruitment into the adult population. Other studies have demonstrated that larval krill have lower lipid stores than adult krill (Hagen *et al.* 2001; Schmidt & Atkinson 2016), can only survive short-term starvation (O'Brien *et al.* 2011; Virtue *et al.* 2016), and are heavily dependent on sea ice for food (sea ice algae) and refuge (Daly 1990). This suggests that reductions in sea ice extent as the climate warms may negatively affect larval krill feeding and their subsequent recruitment to the adult population (Stammerjohn *et al.*, 2008; Massom & Stammerjohn 2010). More adaptable feeding strategies have also been observed in young krill, with substantial levels of flagellate (18:4n-3 and 22:6n-3), carnivory (18:1n-9c/18:1n-7c) and marine snow (C_{15} + C_{17}) biomarkers occurring in larvae

and juveniles collected in winter (Virtue *et al.* 2016). Juvenile krill may also adapt their feeding, consuming more sea ice algae when sea ice is more abundant, and exploiting other food sources in low sea ice years (Bernard *et al.* 2018).

Further studies using sampling regimes at a similar scale to the one used in this study, are needed to examine in detail the diet of krill larvae and juveniles. Additional tools such as gut examination and stable isotope analysis (SIA), including compound specific-SIA, can be also used in conjunction with lipid biochemistry (Schmidt *et al.* 2006; De Troch *et al.* 2012) to more thoroughly examine the diet of krill across all life stages and seasons.

6.3. Krill biochemistry and physiology in a future ocean

The laboratory experiments in this thesis build on a growing volume of long-term studies (Brown 2010; Brown *et al.* 2010, 2013; Höring *et al.* 2018), that are piecing together crucial information on the complex physiology of krill.

In Chapters 4 and 5, it was demonstrated that adult krill appear resilient to near-future ocean acidification (1000 – 2000 µatm pCO_2) across a wide range of physiological and biochemical parameters. Krill survival, length, maturation, respiration rate, haemolymph pH, total lipid, triacylglycerol storage and fatty acid composition were similar in krill reared in ambient seawater (400 µatm pCO_2) and 1000 – 2000 µatm pCO_2 seawater. It was only at 4000 µatm pCO_2 (a level of pCO_2 that krill will not experience in the wild), that negative effects on krill biochemistry and physiology were observed. When the above results are compared with responses of a range of organisms to ocean acidification that have been compiled in meta-analyses (Kroeker *et al.*, 2010; Harvey *et al.* 2013), adult krill are among the most resilient organisms exposed to ocean acidification to date. These results and those obtained by Brown

(2010), who found little effect of elevated temperature on krill, are promising for the future of krill in a high CO_2 world.

The endogenous rhythms that control physiological processes in krill, however, may influence their ability to adapt to climate change. Growth, feeding, lipid deposition, maturation and respiration in krill are partially controlled by an endogenous timing system (biological clock) that is also cued by the seasonal light regime (Meyer *et al.* 2010; Höring *et al.* 2018). This innate timing system is an advantage in the environment that krill have evolved in, but it may decrease their phenotypic plasticity in an ocean that is rapidly changing. The ability of krill to override or alter this timing system on exposure to environmental stressors and low food supply will be a key area for future research.

As stated in Chapter 4, the early life stages of krill (eggs and embryos) are more vulnerable to elevated pCO_2 (Kawaguchi *et al.* 2011, 2013). Krill egg hatch rates decrease above 1250 µatm pCO_2 (Kawaguchi *et al.* 2013) and embryonic development is disrupted at levels of 2000 µatm pCO_2 (Kawaguchi *et al.* 2011). Increasing ocean pCO_2 and temperature will also occur in synergy, and at the present time there are no published studies on the effects of combined warming and acidification on krill. A multi-stressor approach was not possible for this thesis, as such experiments require extensive space and resources that were not available. Multi-stressor experiments will, however, be important for krill research moving forward, and this has been identified as a priority for marine climate change studies in general (Boyd *et al.* 2014; Riebesell & Gattuso 2014). Another key focus for future studies will be to investigate whether adult krill resilient to high pCO_2 are able to produce offspring that are more resilient to high pCO_2. This has been demonstrated in other species (Suckling *et al.* 2015; Ross *et al.* 2016; Wong *et al.* 2018), and will impact the way we assess the vulnerability of early life stages of krill.

6.4. Further application of the research

Data collected during this thesis has a broad range of commercial, ecological and modelling applications. Fatty acid data is not only ecologically useful to assess krill and ecosystem health, but also commercially useful for the krill fishery who harvest krill for their omega-3 fatty acids (primarily EPA and DHA). Results from Chapter 2 have recently been taken up by the krill fishery in an application to change the nutraceutical specifications for krill oil supplements (Aker Biomarine, personal communication). The extreme seasonality and regionality of the fatty acid profile presented in Chapter 2 shows that in some seasons and regions, percentages of specific fatty acids are outside monograph specifications. This application is currently in progress and if accepted, will significantly decrease the percentage of krill oil that is outside specifications and deemed unusable.

The fatty acid data obtained in this study may also contribute to the management of the krill fishery. One specific example is that the fatty acid data in Chapter 2 shows that quantities of EPA and DHA are consistently highest in krill during autumn, at the South Orkney Islands (SOI) and West Antarctic Peninsula (WAP). It is reasonable to assume that the fishery may choose to increase fishing effort during autumn in order to maximise omega-3 harvest. From an ecological perspective, the WAP region contains high concentrations of krill predators (Kawaguchi *et al.* 2009), and the breeding season of predators and development of their young occurs during summer and into early autumn (Reid & Arnould 1996; Croxall *et al.* 1999). The breeding success of many krill predators is directly linked to krill abundance (Lynnes *et al.* 2004; Trivelpiece *et al.* 2011), and an overlap between increased fishing and predation pressure in autumn may have negative consequences for the ecosystem. Seasonal fluctuations in krill lipid content, flow-on effects to higher predators, and the interaction with seasonal harvesting of krill, should be parameterised in CCAMLR fisheries models. Potential effects of climate change on the krill fishery are also not currently considered in these models (Kock *et al.* 2007;

Kawaguchi *et al.* 2009) due to a paucity of data. As fisheries models become more advanced, they could be updated using lipid and fatty acid data obtained from the present study, and additional krill lipid and physiological data as it is collected. This will aid predictions of krill biomass and guide fishing quotas, as fishing effort increases and oceanic climate change occurs.

The use of biochemical and physiological data (obtained from wild and experimental krill) in krill-specific models has been limited to date. Krill egg hatching success in ocean acidification experiments has been used to simulate risk maps for krill eggs in a future high pCO_2 ocean (Kawaguchi *et al.* 2013), and models of krill growth and reproduction have been developed (Brown *et al.* 2010; Constable & Kawaguchi 2018). Krill total lipid data collected by Hagen *et al.* (2001) has been incorporated into a Dynamic Energy Budget (DEB) model for krill (Jager & Ravagnan 2015). DEB models are parameterised using a range of physiological data (e.g. growth, reproduction, maturation, and feeding) with the aim of understanding how these processes affect each other across all life stages, and how an organism's physiology responds to stressors (Jager *et al.* 2013). The DEB model for krill in Jager and Ravagnan (2015) has some limitations and uncertainties, and has very limited data for lipid storage. The lipid data obtained in this thesis could be used to further update this model. It was outside the scope of this thesis to parameterise a new DEB model for krill, but such a model would be the ideal platform for the data collected in this study. Furthermore, DEB models that simulate the effects of ocean acidification on krill physiology at all life stages could also be developed using the data obtained in this thesis.

Ecosystem models are used at a larger scale to simulate and link a range of processes such as biomass, recruitment, reproductive success and trophic interactions within an ecosystem (Fennel & Neumann 2015). They can also further our understanding of how

anthropogenic stressors (e.g. fishing and increasing CO_2 emissions) will synergistically affect whole ecosystems (Griffith *et al.* 2012; Olsen *et al.* 2018). Krill have been included in a wide range of Antarctic ecosystem models (Murphy 1995; Hill *et al.* 2006; Melbourne-Thomas *et al.* 2013; Constable *et al.* 2016), due to their prominent role in the food web. The incorporation of krill lipid data into ecosystem models would be useful to estimate the transfer of lipid energy from krill to higher predators. When lipid energy is modelled in other species, it is closely correlated with reproductive success and recruitment (Marshall *et al.* 1999). The utility of biochemical tracers, such as fatty acids, in quantitative and qualitative models is being increasingly acknowledged (Iverson *et al.* 2004; Pethybridge *et al.* 2015, 2018). Combined total lipid and fatty acid data are especially powerful, as they provide information on both energy provision and diet, which are useful predictors of overall ecosystem health and trophic structure.

The incorporation of biochemical data into ecosystem models is difficult, however, as fatty acid data is often obtained from samples collected at small temporal or spatial scales, or is heavily weighted towards organisms from lower trophic levels (Pethybridge *et al.* 2018). The large spatial and temporal coverage of the data obtained in this thesis could contribute significantly to improve model parameters for krill diet and the Southern Ocean food-web. Laboratory data on effects of ocean acidification on other species is being used to guide models (Busch & McElhany 2016; Fay *et al.* 2017; Olsen *et al.* 2018) and this will be increasingly possible for krill as more experimental data becomes available.

6.5. Conclusions

As a collective body of work, this thesis highlights the remarkable adaptability of krill in a changing environment, whether these changes are at short-term seasonal or annual scales, or on long-term climate change scales. Their flexible phenotype may aid their survival in an ocean

that is rapidly changing, including due to increasing anthropogenic CO_2 emissions. However, the endogenous rhythms controlling their physiology may impair their ability to respond to levels of seawater pCO_2 and temperature they have not experienced before. The data obtained in this thesis also has a wide range of applications in fisheries management and modelling, and has implications for the health of the wider Southern Ocean ecosystem. Further krill lipid and physiological research is needed to build on the present study, and to establish how krill will fare into the future.

Appendices

Appendix I

A. Fatty acid composition (% of total fatty acids ± SD) determined by GC-FID, total lipid content (% DM ± SD) and sum of polyunsaturated, monounsaturated, and saturated fatty acids (PUFA, MUFA, SFA) (% ± SD) of male and female *Euphausia superba* caught in summer, autumn, winter and spring of 2014. Fishing locations: WAP = West Antarctic Peninsula, SOI = South Orkney Islands, SG = South Georgia.

Fishing Year One (2014)								
	Summer : WAP + SOI		Autumn : SOI		Winter : SG + SOI		Spring : SG	
FATTY ACID	Females (*n = 26)*	Males *(n = 20)*	Females *(n = 20)*	Males *(n = 22)*	Females *(n = 22)*	Males *(n = 20)*	Females *(n = 3)*	Males *(n = 3)*
14:0	**5.4** ± 3.0	**5.0** ± 2.9	**9.8** ± 1.6	**11.2** ± 1.5	**9.7** ± 1.2	**9.2** ± 1.3	**9.2** ± 0.3	**8.7** ± 0.5
16:4n-1	**0.4** ± 0.4	**0.6** ± 0.5	**0.7** ± 0.5	**0.7** ± 0.3	**1.1** ± 0.5	**1.3** ± 0.4	**0.7** ± 0.4	**0.9** ± 0.3
16:1n-7c	**4.6** ± 2.6	**4.1** ± 1.7	**8.1** ± 1.5	**9.1** ± 2.0	**6.8** ± 1.1	**6.5** ± 1.4	**6.9** ± 2.0	**6.7** ± 0.5
16:0	**18.3** ± 2.6	**17.1** ± 2.0	**21.8** ± 1.7	**22.1** ± 1.8	**20.8** ± 1.1	**20.4** ± 1.4	**20.9** ± 0.6	**20.5** ± 0.3
18:4n-3	**2.6** ± 1.8	**2.6** ± 1.0	**2.0** ± 1.0	**2.3** ± 1.5	**3.5** ± 1.4	**4.4** ± 2.1	**1.7** ± 1.1	**1.5** ± 0.5
18:2n-6	**2.1** ± 0.6	**2.3** ± 0.6	**1.7** ± 0.5	**1.6** ± 0.3	**1.8** ± 0.3	**1.8** ± 0.3	**1.7** ± 0.2	**1.7** ± 0.1
18:3n-3	**1.5** ± 0.9	**1.6** ± 0.7	**0.7** ± 0.2	**0.7** ± 0.4	**1.2** ± 0.5	**1.4** ± 0.7	**0.7** ± 0.3	**0.6** ± 0.3
18:1n-9c	**11.0** ± 2.7	**9.3** ± 2.0	**13.7** ± 1.4	**13.5** ± 1.4	**11.5** ± 2.3	**10.7** ± 2.6	**13.7** ± 2.0	**13.3** ± 1.5
18:1n-7c	**7.8** ± 1.1	**7.0** ± 0.9	**8.0** ± 1.0	**7.2** ± 0.5	**6.1** ± 0.8	**5.7** ± 0.7	**6.4** ± 0.7	**6.8** ± 0.4
18:0	**1.1** ± 0.3	**1.0** ± 0.3	**1.3** ± 0.2	**1.4** ± 0.2	**1.5** ± 0.3	**1.4** ± 0.3	**1.7** ± 0.2	**1.6** ± 0.2
20:5n-3	**20.0** ± 4.3	**21.3** ± 4.5	**15.7** ± 2.2	**14.9** ± 2.0	**16.0** ± 1.8	**16.2** ± 1.4	**16.4** ± 0.8	**17.7** ± 1.2
20:1n-9c	**0.7** ± 0.3	**0.5** ± 0.3	**1.0** ± 0.3	**1.0** ± 0.2	**0.7** ± 0.2	**0.7** ± 0.2	**0.9** ± 0.1	**0.9** ± 0.2
21:5n-3	**0.5** ± 0.2	**0.6** ± 0.2	**0.4** ± 0.1	**0.4** ± 0.1	**0.5** ± 0.1	**0.6** ± 0.1	**0.4** ± 0.1	**0.5** ± 0.1
22:6n-3	**16.4** ± 7.6	**19.3** ± 7.0	**7.9** ± 2.2	**6.8** ± 1.5	**9.1** ± 1.4	**9.7** ± 1.9	**10.2** ± 1.9	**10.9** ± 0.9
22:1n-9c	**0.4** ± 0.2	**0.6** ± 0.4	**0.8** ± 0.3	**0.8** ± 0.2	**0.6** ± 0.2	**0.5** ± 0.2	**1.0** ± 0.3	**0.9** ± 0.3
Other*	**7.1** ± 1.6	**7.4** ± 1.4	**6.5** ± 1.0	**6.2** ± 0.8	**9.2** ± 1.9	**9.6** ± 2.0	**7.6** ± 1.4	**6.9** ± 0.4
SUM PUFA	**46.1** ± 11.8	**49.6** ± 11.4	**31.1** ± 5.2	**29.4** ± 5.3	**35.2** ± 4.2	**37.4** ± 5.2	**33.7** ± 2.2	**35.8** ± 2.9
SUM MUFA	**27.4** ± 6.4	**24.9** ± 5.7	**34.5** ± 2.8	**34.5** ± 3.2	**29.4** ± 3.5	**27.8** ± 4.0	**31.9** ± 2.5	**31.8** ± 2.6
SUM SFA	**26.1** ± 5.8	**25.1** ± 5.7	**34.3** ± 3.1	**36.1** ± 2.8	**33.6** ± 2.3	**32.7** ± 2.5	**33.2** ± 0.9	**32.4** ± 0.6
Total Lipid (% DM)	**10.2** ± 6.5	**8.7** ± 6.0	**26.9** ± 10.1	**33.0** ± 6.8	**20.4** ± 6.6	**21.6** ± 8.5	**11.5** ± 3.0	**6.0** ± 1.3

* Other fatty acids <0.5% of total fatty acids: 14:1, *i*16:0, 16:0 FALD, 16:3, 16:1n-5, 16:1n-9c, 16:1n-13, 18:0, *i*18:0, 18:1, 18:1n-7t, 18:1n-5, 18:3ω6, 20:0, 20:2n-6, 20:3n-6, 20:4n-3, 20:4n-6, C20 PUFAs, 21:0, 22:0, 22:4n-6, 22:5n-3, 22:5n-6, 24:0, 24:1n-7c, 24:1n-9c, 24:1n-11c, phytanic acid. Limit of detection (LOD) was determined to be 0.05% or 0.5 ng. Similar LOD occurred for GC-MS analyses and compositional results.

B. Fatty acid composition (% of total fatty acids ± SD) determined by GC-FID, total lipid content (% DM ± SD) and sum of polyunsaturated, monounsaturated, and saturated fatty acids (PUFA, MUFA, SFA) (% ± SD) of male and *female Euphausia superba* caught in summer, autumn, winter and spring of 2015. Fishing locations: WAP = West Antarctic Peninsula, SOI = South Orkney Islands, SG = South Georgia.

Fishing Year Two (2015)								
	Summer : WAP + SOI		Autumn : WAP		Winter : SG		Spring : SG	
FATTY ACID	Females *(n = 18)*	Males *(n = 16)*	Females *(n = 21)*	Males *(n = 20)*	Females *(n = 18)*	Males *(n = 18)*	Females *(n = 6)*	Males *(n = 6)*
14:0	**8.3** ± 1.9	**9.3** ± 2.1	**9.4** ± 1.0	**9.2** ± 1.1	**9.2** ± 1.4	**9.0** ± 1.4	**7.9** ± 1.0	**7.9** ± 1.3
16:4n-1	**0.8** ± 0.7	**1.3** ± 0.9	**1.1** ± 0.3	**1.2** ± 0.4	**0.9** ± 0.5	**1.1** ± 0.4	**0.5** ± 0.2	**0.7** ± 0.4
16:1n-7c	**6.4** ± 2.1	**7.3** ± 1.8	**5.5** ± 1.5	**5.2** ± 1.9	**8.3** ± 1.1	**7.7** ± 0.9	**7.0** ± 0.4	**7.0** ± 0.9
16:0	**20.1** ± 1.3	**21.0** ± 1.0	**20.3** ± 1.3	**19.8** ± 1.5	**22.3** ± 1.0	**22.2** ± 0.9	**21.6** ± 0.4	**21.2** ± 0.7
18:4n-3	**3.2** ± 1.0	**3.6** ± 1.2	**4.7** ± 1.7	**6.0** ± 1.8	**1.7** ± 0.9	**2.0** ± 0.7	**1.1** ± 0.2	**1.2** ± 0.2
18:2n-6	**1.7** ± 0.5	**1.5** ± 0.4	**1.5** ± 0.3	**1.6** ± 0.2	**1.4** ± 0.4	**1.3** ± 0.4	**1.9** ± 0.4	**1.6** ± 0.2
18:3n-3	**1.3** ± 0.6	**1.2** ± 0.6	**2.0** ± 0.9	**2.4** ± 0.9	**0.5** ± 0.1	**0.5** ± 0.1	**0.6** ± 0.1	**0.5** ± 0.1
18:1n-9c	**12.2** ± 2.3	**12.8** ± 1.8	**11.6** ± 2.0	**11.0** ± 1.2	**14.2** ± 1.4	**13.7** ± 0.9	**14.8** ± 1.0	**13.4** ± 1.2
18:1n-7c	**7.5** ± 1.1	**6.4** ± 0.9	**7.3** ± 0.8	**6.8** ± 0.5	**7.1** ± 0.6	**6.7** ± 0.4	**7.2** ± 0.5	**6.6** ± 0.5
18:0	**1.2** ± 0.3	**1.5** ± 0.5	**1.2** ± 0.3	**1.1** ± 0.2	**1.5** ± 0.2	**1.7** ± 0.3	**1.5** ± 0.1	**1.7** ± 0.3
20:5n-3	**18.4** ± 1.8	**17.5** ± 1.9	**17.4** ± 1.7	**17.2** ± 1.5	**15.6** ± 1.4	**16.5** ± 1.8	**16.5** ± 1.2	**18.9** ± 2.4
20:1n-9c	**0.7** ± 0.2	**0.8** ± 0.2	**0.6** ± 0.3	**0.6** ± 0.2	**1.0** ± 0.2	**1.0** ± 0.2	**0.9** ± 0.3	**1.0** ± 0.2
21:5n-3	**0.6** ± 0.2	**0.7** ± 0.3	**0.6** ± 0.1	**0.7** ± 0.1	**0.5** ± 0.2	**0.6** ± 0.2	**0.4** ± 0.1	**0.5** ± 0.1
22:6n-3	**10.8** ± 3.7	**8.8** ± 2.2	**9.2** ± 1.8	**9.8** ± 1.3	**8.1** ± 1.4	**8.5** ± 1.2	**9.8** ± 0.9	**10.1** ± 0.5
22:1n-9c	**0.4** ± 0.2	**0.7** ± 0.2	**0.5** ± 0.3	**0.6** ± 0.2	**0.9** ± 0.3	**0.8** ± 0.2	**0.8** ± 0.2	**0.8** ± 0.3
Other*	**6.5** ± 1.8	**5.7** ± 1.1	**7.0** ± 1.4	**7.0** ± 0.7	**6.6** ± 0.7	**6.6** ± 0.8	**7.6** ± 1.5	**7.1** ± 1.3
SUM PUFA	**38.9** ± 6.0	**36.5** ± 5.4	**38.5** ± 4.8	**40.9** ± 3.7	**30.6** ± 3.6	**32.3** ± 3.3	**32.7** ± 1.8	**35.6** ± 3.4
SUM MUFA	**30.1** ± 3.9	**30.6** ± 2.9	**29.0** ± 3.3	**27.5** ± 2.4	**34.8** ± 2.6	**33.2** ± 1.9	**34.1** ± 1.4	**31.8** ± 2.1
SUM SFA	**30.8** ± 2.8	**32.8** ± 2.9	**32.2** ± 2.1	**31.4** ± 1.8	**34.6** ± 1.8	**34.5** ± 1.7	**32.7** ± 1.3	**32.3** ± 1.8
Total Lipid (% DM)	**20.1** ± 9.2	**23.9** ± 7.3	**30.4** ± 7.4	**32.3** ± 6.1	**23.3** ± 6.4	**23.4** ± 6.1	**13.1** ± 1.7	**13.9** ± 1.7

* Other fatty acids and GC-FID limit of detection, see Appendix IA.

C. Fatty acid composition (% of total fatty acids ± SD) determined by GC-FID, total lipid content (% DM ± SD) and sum of polyunsaturated, monounsaturated, and saturated fatty acids (PUFA, MUFA, SFA) (% ± SD) of male and female *Euphausia superba* caught in summer, autumn, winter and spring of 2016. Fishing locations: WAP = West Antarctic Peninsula, SOI = South Orkney Islands, SG = South Georgia.

Fishing Year Three (2016)								
	Summer : WAP + SOI		Autumn : WAP		Winter : SG		Spring : SG	
FATTY ACID	Females *(n = 22)*	Males *(n = 21)*	Females *(n = 19)*	Males *(n = 18)*	Females *(n = 21)*	Males *(n = 18)*	Females *(n = 5)*	Males *(n = 6)*
14:0	**7.7** ± 2.3	**9.2** ± 2.6	**11.3** ± 1.3	**12.2** ± 1.3	**11.6** ± 1.6	**11.6** ± 0.7	**10.1** ± 1.4	**9.5** ± 1.7
16:4n-1	**1.1** ± 0.4	**1.3** ± 0.6	**1.1** ± 0.4	**1.2** ± 0.4	**0.5** ± 0.2	**0.6** ± 0.2	**0.4** ± 0.1	**0.3** ± 0.1
16:1n-7c	**6.8** ± 1.8	**7.0** ± 1.6	**9.1** ± 1.5	**9.5** ± 1.1	**9.6** ± 1.2	**9.3** ± 0.8	**8.2** ± 1.0	**7.6** ± 0.9
16:0	**20.4** ± 1.6	**20.9** ± 2.1	**22.4** ± 1.5	**23.3** ± 0.9	**22.8** ± 0.9	**22.8** ± 1.1	**22.3** ± 0.1	**21.6** ± 0.7
18:4n-3	**3.3** ± 1.3	**3.8** ± 1.3	**2.2** ± 0.7	**2.2** ± 0.6	**1.2** ± 0.4	**1.5** ± 0.7	**1.1** ± 0.1	**1.0** ± 0.4
18:2n-6	**1.2** ± 0.4	**1.2** ± 0.4	**1.0** ± 0.6	**0.8** ± 0.4	**1.8** ± 0.3	**1.7** ± 0.4	**1.9** ± 0.5	**1.9** ± 0.2
18:3n-3	**1.1** ± 0.9	**1.1** ± 0.9	**0.6** ± 0.3	**0.5** ± 0.2	**0.6** ± 0.2	**0.7** ± 0.2	**0.6** ± 0.1	**0.7** ± 0.1
18:1n-9c	**12.3** ± 2.2	**12.3** ± 2.5	**14.5** ± 1.5	**14.8** ± 1.2	**14.5** ± 1.5	**13.9** ± 2.3	**15.1** ± 1.8	**15.1** ± 1.9
18:1n-7c	**7.1** ± 1.3	**6.2** ± 0.7	**7.0** ± 1.1	**6.2** ± 0.6	**7.3** ± 0.9	**7.0** ± 0.9	**6.9** ± 0.4	**7.1** ± 0.3
18:0	**1.5** ± 0.4	**1.6** ± 0.3	**1.4** ± 0.2	**1.5** ± 0.2	**1.5** ± 0.2	**1.5** ± 0.2	**1.5** ± 0.2	**1.5** ± 0.2
20:5n-3	**19.2** ± 3.1	**19.0** ± 3.7	**16.2** ± 2.5	**15.6** ± 2.2	**13.6** ± 1.3	**14.0** ± 0.6	**14.7** ± 1.3	**15.6** ± 1.8
20:1n-9c	**0.8** ± 0.3	**0.8** ± 0.3	**1.1** ± 0.3	**1.3** ± 0.2	**1.1** ± 0.2	**1.0** ± 0.3	**1.1** ± 0.3	**1.0** ± 0.2
21:5n-3	**0.8** ± 0.1	**0.8** ± 0.3	**0.6** ± 0.2	**0.7** ± 0.2	**0.4** ± 0.1	**0.4** ± 0.1	**0.4** ± 0.1	**0.4** ± 0.1
22:6n-3	**10.0** ± 3.6	**8.9** ± 3.8	**6.0** ± 1.9	**5.2** ± 1.7	**6.3** ± 1.2	**6.4** ± 0.9	**8.2** ± 0.8	**9.4** ± 1.9
22:1n-9c	**0.5** ± 0.2	**0.8** ± 0.3	**0.9** ± 0.3	**1.0** ± 0.2	**0.9** ± 0.2	**0.8** ± 0.3	**0.9** ± 0.2	**0.8** ± 0.2
Other*	**6.3** ± 1.8	**5.5** ± 1.3	**4.8** ± 1.1	**4.2** ± 0.7	**6.5** ± 1.1	**6.7** ± 1.4	**6.8** ± 1.5	**6.7** ± 1.3
SUM PUFA	**39.0** ± 6.6	**37.9** ± 8.0	**29.0** ± 4.6	**27.2** ± 3.7	**26.1** ± 3.0	**27.2** ± 2.6	**29.1** ± 2.0	**31.2** ± 3.7
SUM MUFA	**30.0** ± 3.8	**29.3** ± 4.1	**34.9** ± 2.7	**34.9** ± 1.9	**36.3** ± 1.6	**35.0** ± 2.1	**35.1** ± 2.2	**34.4** ± 2.2
SUM SFA	**30.8** ± 3.9	**32.7** ± 4.4	**36.1** ± 2.3	**37.9** ± 2.1	**37.5** ± 2.2	**37.5** ± 1.3	**35.4** ± 1.5	**34.2** ± 2.4
Total Lipid (% DM)	**18.0** ± 10.2	**22.2** ± 13.5	**36.1** ± 9.7	**40.0** ± 5.2	**20.4** ± 5.9	**20.1** ± 4.6	**11.9** ± 1.0	**9.1** ± 2.7

* Other fatty acids and GC-FID limit of detection, see Appendix IA.

Appendix II

Interannual differences in the composition (% of total fatty acids, mean ± SD) of polyunsaturated (PUFA), monounsaturated (MUFA) and saturated fatty acids (SFA) in *Euphausia superba* collected in summer, autumn, winter and spring during the different fishing years. Significant differences among years are denoted by cells that do not share lower case letters, and *p* values are stated for significant differences. When there is more than one significant difference among years, the highest *p* value is stated.

Summer				
Fatty Acid Class	**2014**	**2015**	**2016**	***p* value**
PUFA	47.6 ± 11.5 ^{a}	37.8 ± 5.7 ^{b}	38.4 ± 7.3 ^{b}	< 0.001
MUFA	26.3 ± 6.1 ^{a}	30.3 ± 3.4 ^{b}	29.6 ± 3.9 ^{b}	< 0.004
SFA	25.7 ± 5.7 ^{a}	31.8 ± 3.0 ^{b}	31.7 ± 4.2 ^{b}	< 0.001
Autumn				
Fatty Acid Class	**2014**	**2015**	**2016**	***p* value**
PUFA	30.2 ± 5.3 ^{a}	39.7 ± 4.4 ^{b}	28.1 ± 4.2 ^{a}	< 0.001
MUFA	34.5 ± 3.0 ^{a}	28.2 ± 3.0 ^{b}	35.0 ± 2.3 ^{a}	< 0.001
SFA	35.3 ± 3.0 ^{a}	31.8 ± 1.9 ^{b}	37.0 ± 2.4 ^{c}	< 0.004
Winter				
Fatty Acid Class	**2014**	**2015**	**2016**	***p* value**
PUFA	36.3 ± 4.8 ^{a}	31.4 ± 3.5 ^{b}	26.5 ± 2.8 ^{c}	< 0.001
MUFA	28.6 ± 3.8 ^{a}	34.0 ± 2.4 ^{b}	35.7 ± 2.0 ^{c}	< 0.016
SFA	33.2 ± 2.4 ^{a}	34.6 ± 1.8 ^{b}	37.5 ± 1.8 ^{c}	< 0.009
Spring				
Fatty Acid Class	**2014**	**2015**	**2016**	***p* value**
PUFA	34.7 ± 3.1 ^{a}	34.2 ± 3.0 ^{a}	30.1 ± 3.0 ^{b}	< 0.011
MUFA	31.9 ± 2.3 ^{a}	32.9 ± 2.1 ^{ac}	34.7 ± 2.0 ^{c}	< 0.030
SFA	32.8 ± 0.8 ^{a}	32.5 ± 1.5 ^{a}	34.9 ± 2.0 ^{b}	< 0.053

Appendix III

Eigenvalues, variation (%) cumulative variation (%) and loadings for principal component analysis of fatty acid percentage composition data of *Euphausia superba*. Largest loadings for positive and negative components of each principal component (PC1 and PC2) are highlighted in bold.

	2014		**2015**		**2016**	
Principal Component	**PC1**	**PC2**	**PC1**	**PC2**	**PC1**	**PC2**
Eigenvalue	0.745	0.232	0.426	0.128	0.353	0.169
Variation (%)	64.4	20.1	60.4	18.2	51.8	24.8
Cumulative Variation (%)	-	84.4	-	78.6	-	76.6
Loadings						
14:0	**-0.526**	0.215	-0.050	0.077	**-0.346**	0.100
16:0	-0.131	-0.010	-0.072	0.075	-0.114	0.041
16:1n-7c	-0.497	0.013	**-0.339**	0.284	-0.324	0.100
16:4n-1	-0.142	0.415	0.134	**0.628**	0.177	**0.489**
18:0	-0.118	0.055	-0.125	0.229	-0.035	0.070
18:1n-7c	-0.025	**-0.197**	-0.015	-0.156	-0.008	-0.157
18:1n-9c	-0.191	-0.131	-0.163	0.153	-0.214	0.066
18:2n-6	0.123	-0.018	0.045	**-0.361**	0.063	**-0.532**
18:3n-3	0.260	0.280	0.523	-0.050	0.307	-0.147
18:4n-3	0.067	**0.780**	**0.673**	0.288	**0.464**	0.457
20:1n-9c	-0.144	-0.130	-0.175	0.163	-0.223	0.125
20:5n-3	0.171	-0.027	0.044	-0.064	0.223	0.114
21:5n-3	0.041	0.099	0.077	0.229	0.150	0.217
22:1n-9c	-0.175	-0.089	-0.174	0.120	-0.248	0.075
22:6n-3	**0.467**	-0.006	0.140	-0.316	0.432	-0.335

Appendix IV

Absolute quantities of total lipid (mg g $^{-1}$ dry mass; DM), and percentages of triacylglycerol (TAG) and phospholipid (PL) (as % of total lipid, obtained using Iatroscan TLC-FID analyser) in male *Euphausia superba* samples chosen for lipid class fatty acid analysis. The seasons (summer = Jan and Feb; autumn = Mar – May; winter/spring = Jun – Sep) and locations (SOI = South Orkney Islands; WAP = West Antarctic Peninsula; SG = South Georgia) in which the krill were caught are also shown. All samples were collected in 2016 by the fishing vessel *FV Saga Sea*.

Krill I.D.	Season	Location	Total Lipid (mg g $^{-1}$ DM)	TAG (%)	PL (%)
1	Summer	SOI	151.1	38.6	55.4
2	Summer	SOI	124.5	34.4	57.0
3	Summer	SOI	140.3	38.6	48.5
4	Summer	SOI	84.6	34.8	54.5
5	Summer	SOI	317.1	46.3	48.6
6	Summer	SOI	392.4	40.8	55.2
7	Summer	SOI	330.3	41.9	53.1
8	Summer	SOI	381.9	47.6	47.8
9	Summer	SOI	442.7	46.6	46.4
10	Summer	WAP	422.8	49.5	47.3
11	Summer	WAP	336.7	46.6	49.9
12	Summer	WAP	251.7	42.5	47.2
13	Autumn	WAP	369.9	48.0	47.5
14	Autumn	WAP	346.7	46.9	48.8
15	Autumn	WAP	375.0	47.4	46.9
16	Autumn	WAP	433.6	45.8	48.1
17	Autumn	WAP	436.0	46.3	49.0
18	Autumn	WAP	464.7	38.2	41.6
19	Autumn	WAP	428.1	47.4	46.9
20	Autumn	WAP	395.2	45.6	49.5
21	Autumn	WAP	441.2	49.7	46.7
22	Autumn	WAP	324.0	51.3	44.3
23	Autumn	WAP	352.1	51.5	45.5
24	Autumn	WAP	396.1	46.4	48.4
25	Winter/Spring	SG	291.7	46.8	49.7
26	Winter/Spring	SG	215.1	41.8	52.0
27	Winter/Spring	SG	233.8	44.3	52.9
28	Winter/Spring	SG	292.0	43.3	54.1
29	Winter/Spring	SG	223.5	36.6	61.5
30	Winter/Spring	SG	182.0	41.1	55.9
31	Winter/Spring	SG	185.3	43.2	53.6
32	Winter/Spring	SG	171.9	42.9	49.6
33	Winter/Spring	SG	180.3	39.7	57.6
34	Winter/Spring	SG	148.8	36.1	61.2
35	Winter/Spring	SG	131.7	34.0	63.8
36	Winter/Spring	SG	99.2	26.1	71.0

Appendix V

Measured pH_{NIST} (mean ± SD), pH_{total} (mean), pCO_2 (mean), seawater temperature (mean ± SD), alkalinity (mean ± SD), dissolved inorganic carbon (mean ± SD), salinity (mean ± SD), and calcite (Ω_C) and aragonite (Ω_A) saturation of seawater in each pCO_2 treatment during the 46-week experimental period (January – December 2016). Values with an asterisk were calculated using CO_2SYS (Pierrot *et al.* 2006).

Treatment	**pH_{NIST}** (measured)	**pH_{total}*** (mol/kg SW)	***pCO_2**** (µatm)	**Temp (°C)**	**Alkalinity (A_T)** (µmol/kg SW)	**DIC** (µmol/kg SW)	**Salinity** (PSU)	**Ω_C ***	**Ω_A***
400	8.12 (± 0.05)	8.07	392	0.5 (± 0.2)	2456 (± 61)	2320 (± 52)	35.3 (± 0.6)	2.55	1.60
1000	7.79 (± 0.07)	7.73	898	0.5 (± 0.2)	2456 (± 61)	2432 (± 69)		1.25	0.79
1500	7.61 (± 0.06)	7.54	1430	0.5 (± 0.2)	2455 (± 62)	2491 (± 62)		0.82	0.51
2000	7.48 (± 0.07)	7.44	1831	0.5 (± 0.2)	2466 (± 69)	2537 (± 80)		0.65	0.41
4000	7.09 (± 0.08)	7.09	4123	0.5 (± 0.2)	2456 (± 60)	2690 (± 126)		0.30	0.19

Appendix VI

Light regime used during each month of the one-year ocean acidification experiment on adult *Euphausia superba*. Experimental weeks corresponding to each month are also shown in brackets.

Experimental Month	Time lights on	Time lights off	Maximum Lux
January (Week 1)	03:30	20:30	82
February (Weeks 2 – 5)	24 hr daylight	-	100
March (Weeks 6 – 9)	03:30	20:30	82
April (Weeks 10 – 14)	05:30	18:30	64
May (Weeks 15 – 18)	06:00	18:00	45
June (Weeks 19 – 22)	07:30	16:30	27
July (Weeks 23 – 27)	09:30	14:30	9
August (Weeks 28 – 31)	24 hr darkness	-	0
September (Weeks 32 – 36)	09:30	14:30	9
October (Weeks 37 – 40)	07:30	16:30	27
November (Weeks 41 – 44)	06:00	18:00	45
December (Weeks 45 – 46)	05:30	18:30	64

Appendix VII

Sample sizes (n) for *Euphausia superba* (A) total length data and (B) triacylglycerol data for each pCO_2 treatment and experimental week.

pCO_2 Treatment	Week 1	Week 2	Week 4	Week 5	Week 26	Week 39	Week 41	Week 43	Week 46
(A) Total Length									
400 µatm	4	4	4	5	4	10	9	9	5
1000 µatm	6	4	6	6	5	9	10	10	5
1500 µatm	4	5	5	7	4	9	9	9	5
2000 µatm	4	5	6	5	4	9	9	9	5
4000 µatm	4	4	5	4	5	6	6	6	2
(B) Triacylglycerol									
400 µatm	4	4	4	4	4	5	4	5	n/a
1000 µatm	5	4	4	5	5	4	5	5	n/a
1500 µatm	4	5	4	5	4	4	4	4	n/a
2000 µatm	4	5	5	5	4	4	4	4	n/a
4000 µatm	4	4	5	4	5	3	3	3	n/a

Appendix VIII

Maturity stages of subadult and adult *Euphausia superba* and their associated maturity scores. Modified from Seigel (2016b).

Maturity Stage	Description	Maturity Score
Male		
IIA3	subadult male – petasma is two lobed, wing present	1
IIIA	adult male – petasma fully developed	2
Female		
IIB	subadult female – developing thelycum present, colour is feeble or absent	1
IIIA	adult female – thelycum fully developed, red in colour, no spermatophores present, body not swollen	2
IIID	gravid female – thorax and first and second abdominal segment swollen by enlarged ovary	3

Appendix IX

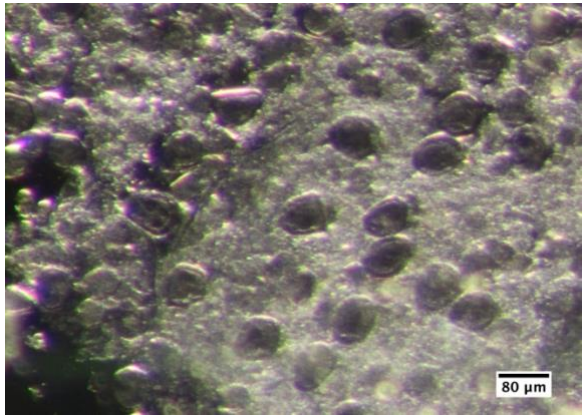
Ovary phases and stages of *Euphausia superba* determined from their cell type, cell size (μm) and squash appearance. Modified from tables and keys in Cuzin-Roudy & Amsler (1991).

Cell Type	Cell Size (μm)	Squash appearance	Physiological Phase	Ovary Stage
yoc	50 - 150	clear	Oogenesis	2
oc1	150 - 200	translucent	Previtellogenesis	3
	200 - 450	translucent	Previtellogenesis	4
oc2	250 - 450	cloudy and whitish	Early Vitellogenesis	5
oc3	over 450	granular and whitish	Vitellogenesis	6

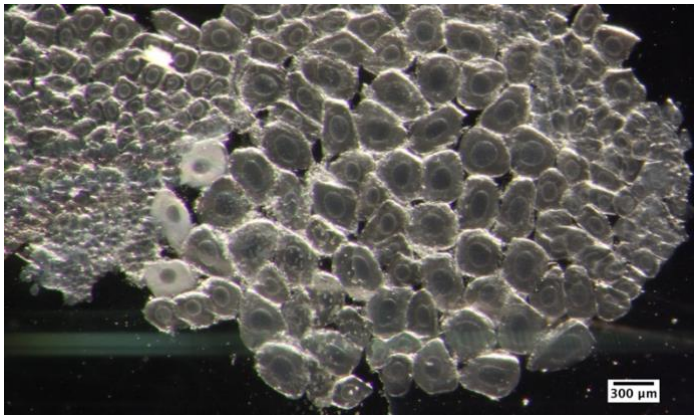
Appendix X

Examples of ovary stages observed in *Euphausia superba*. Ovary stages shown are (**A**) stage 2, oogenesis (**B**) stage 4.5, late previtellogenesis/early previtellogenesis and (**C**) stage 6, vitellogenesis.

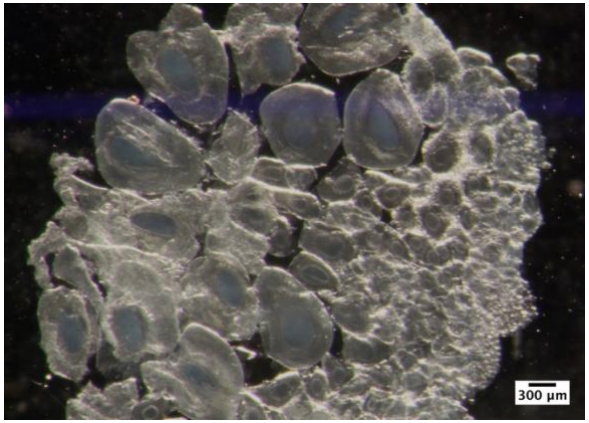
A. Oogenesis



B. Late Previtellogenesis / Early Vitellogenesis



C. Vitellogenesis

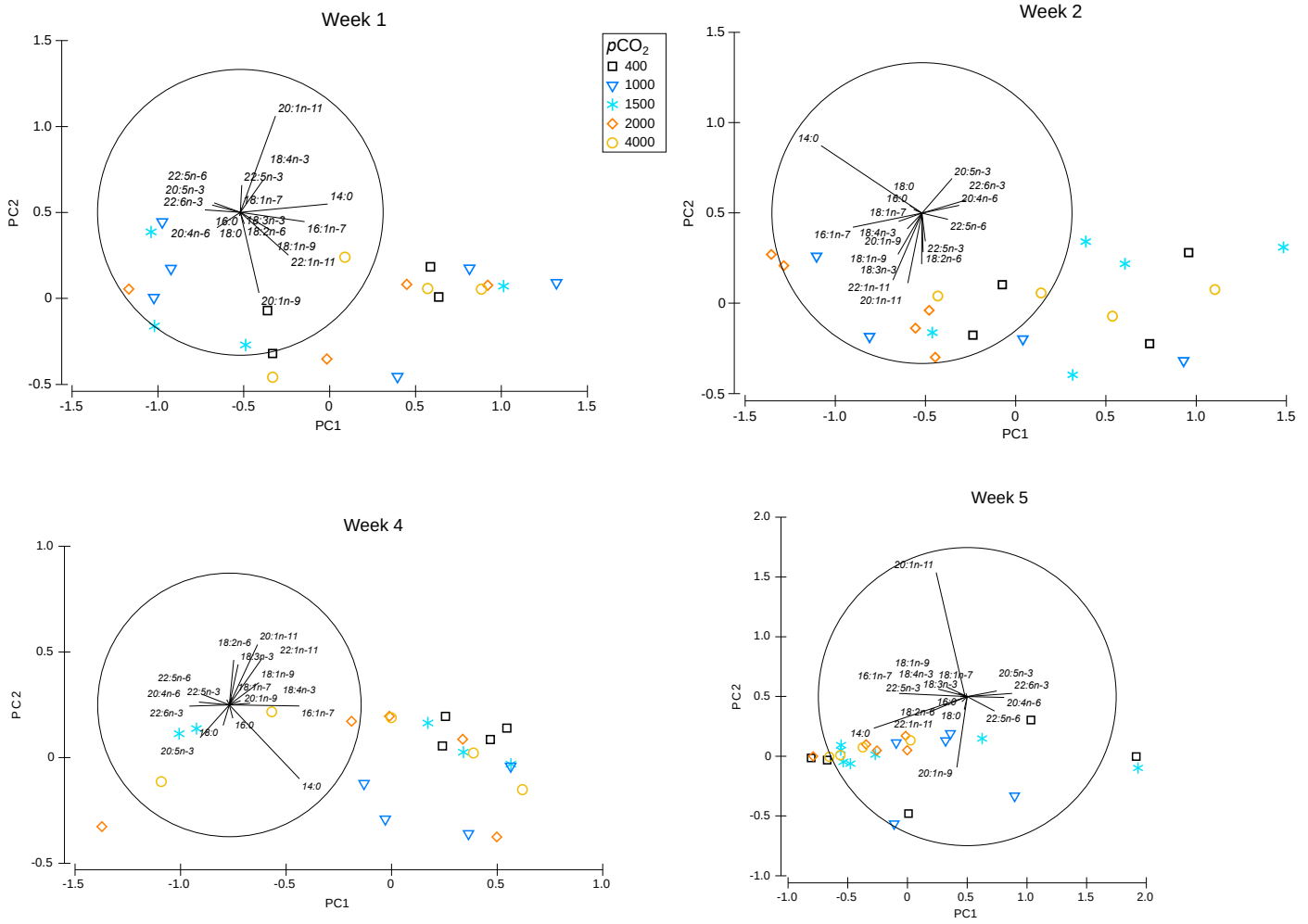


Appendix XI

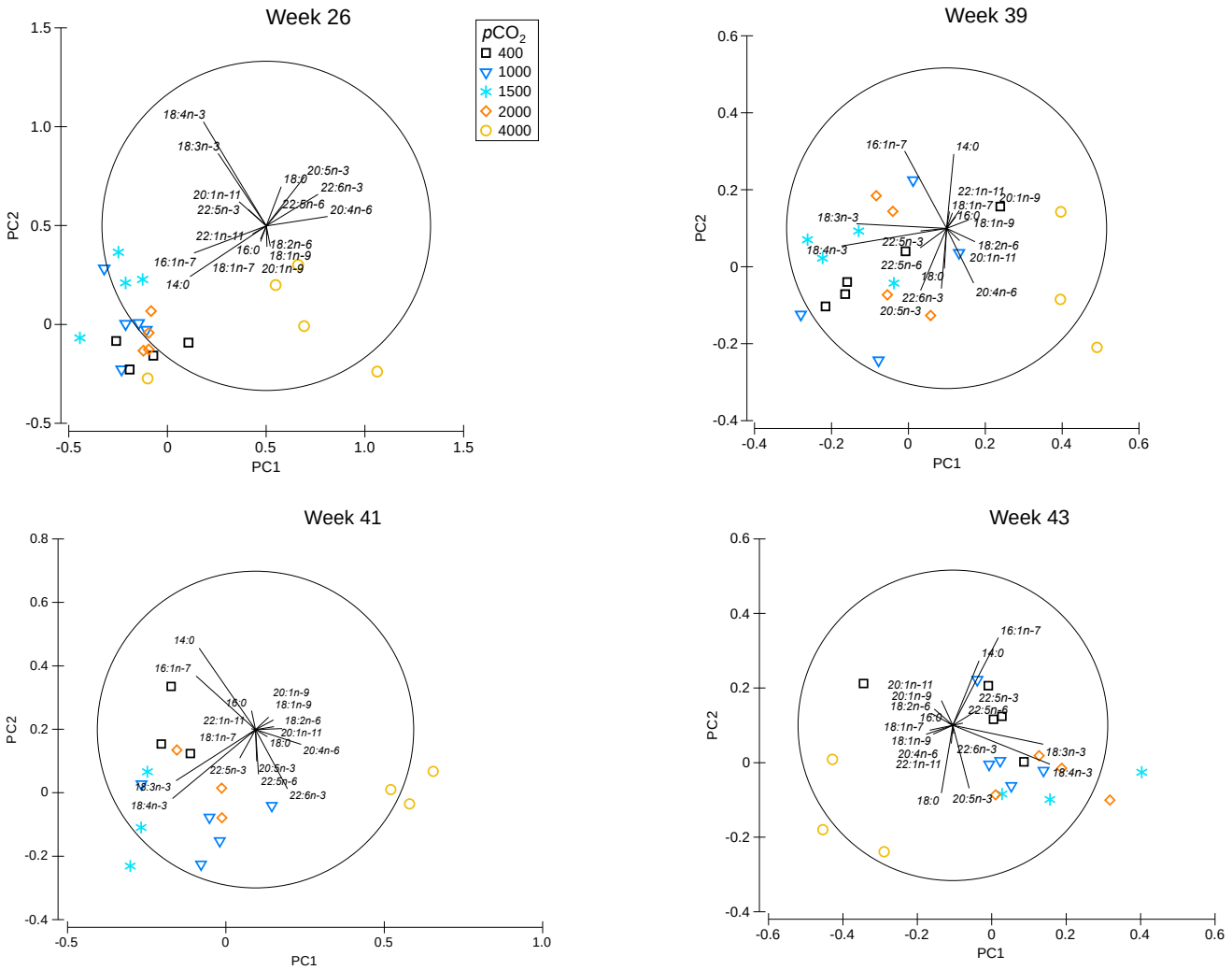
Maturity scores (range and mean) for *Euphausia superba* from each CO_2 treatment, sampled during weeks 39, 41, 43 and 46 (October – December 2016). See Appendix VIII for maturity staging key. For each of the 400, 1000, 1500 and 2000 µatm pCO_2 treatments n = 5, and for the 4000 µatm pCO_2 treatment n = 3.

Treatment (µatm pCO_2)	**Week 39**		**Week 41**		**Week 43**		**Week 46**	
	Range	**Mean**	**Range**	**Mean**	**Range**	**Mean**	**Range**	**Mean**
400	1 – 2	1.6	1 – 2	1.4	1 – 2	1.6	2 – 3	2.2
1000	1 – 2	1.4	1 – 2	1.4	1 – 2	1.4	2	2.0
1500	1 – 2	1.2	1 – 2	1.4	1 – 2	1.6	1 – 2	1.8
2000	1 – 2	1.4	1 – 2	1.4	1 – 2	1.6	1 – 2	1.8
4000	1	1.0	1	1.0	1	1.0	1 – 2	1.5

Appendix XII



Principal component analyses for the fatty acid composition (%) of *Euphausia superba* in separate weeks 1, 2, 4 and 5 of the ocean acidification experiment



Principal component analyses for the fatty acid composition (%) of *Euphausia superba* in separate weeks 26, 39, 41 and 43 of the ocean acidification experiment

Appendix XIII

Percentage variation and cumulative variation, and loadings for principal component analyses of *Euphausia superba* fatty acid composition (%) in combined weeks 1 – 5 and weeks 26 – 43 of the ocean acidification experiment.

	Weeks 1 – 5		**Weeks 26 – 43**	
Principal Component	**PC1**	**PC2**	**PC1**	**PC2**
Variation (%)	80.6	5.4	55.5	20.1
Cumulative Variation (%)	-	86.0	-	75.6
Loadings				
14:0	0.644	0.381	0.398	0.428
16:0	0.036	0.081	0.007	0.090
16:1n-7c	0.471	-0.019	0.426	0.222
18:0	-0.003	0.090	-0.094	-0.162
18:1n-7c	0.007	-0.027	-0.008	0.062
18:1n-9c	0.185	-0.123	-0.079	0.128
18:2n-6	0.023	-0.110	-0.055	0.126
18:3n-3	0.042	-0.123	0.426	-0.382
18:4n-3	0.159	-0.104	0.518	-0.472
20:1n-9c	0.083	0.275	-0.069	0.109
20:1n-11	0.191	-0.804	0.037	0.114
20:4n-6	-0.213	0.009	-0.332	-0.149
20:5n-3	-0.201	0.094	-0.080	-0.379
22:1n-11	0.246	-0.171	0.012	0.060
22:5n-3	-0.004	-0.157	0.134	-0.080
22:5n-6	-0.181	-0.020	-0.075	-0.211
22:6n-3	-0.279	0.008	-0.214	-0.289

Appendix XIV

Percentage composition (mean ± SD) of selected fatty acids in *Euphausia superba* reared in 400, 1000, 1500, 2000 and 4000 pCO_2 seawater in experimental weeks 1, 2, 4 and 5 of the one-year ocean acidification experiment. No significant differences were found between the control treatment (400 µatm pCO_2) and any of the elevated pCO_2 treatments (1000 – 4000 µatm pCO_2) during weeks 1, 2, 4 or 5 ($p > 0.05$). For each pCO_2 treatment $n = 3 – 7$.

Fatty Acid	pCO_2	Week 1	Week 2	Week 4	Week 5
14:0	400	1.35 ± 0.61	1.07 ± 0.72	2.52 ± 0.44	2.48 ± 2.02
	1000	1.30 ± 1.35	2.10 ± 1.91	2.85 ± 0.56	2.38 ± 1.21
	1500	0.90 ± 1.42	1.02 ± 0.87	1.80 ± 1.13	2.83 ± 1.49
	2000	1.50 ± 1.33	3.72 ± 1.86	2.12 ± 1.18	3.26 ± 1.15
	4000	1.45 ± 0.86	1.25 ± 0.91	2.00 ± 1.12	3.52 ± 0.94
16:1n-7c	400	2.73 ± 0.96	2.65 ± 1.07	4.30 ± 0.36	3.86 ± 2.21
	1000	2.75 ± 1.80	3.98 ± 1.74	4.05 ± 1.14	3.16 ± 0.86
	1500	1.98 ± 1.18	2.46 ± 1.01	3.48 ± 1.52	4.17 ± 1.86
	2000	2.65 ± 1.44	5.20 ± 1.22	3.30 ± 1.54	4.54 ± 0.91
	4000	3.05 ± 0.89	2.52 ± 1.02	3.08 ± 1.46	4.65 ± 0.69
18:2n-6	400	7.30 ± 0.52	7.38 ± 0.46	8.00 ± 0.32	7.38 ± 0.63
	1000	7.25 ± 0.82	7.15 ± 0.59	7.05 ± 0.69	7.40 ± 0.37
	1500	7.45 ± 0.31	7.22 ± 0.93	7.56 ± 0.29	7.40 ± 0.41
	2000	7.05 ± 0.37	7.30 ± 0.75	7.32 ± 0.89	7.58 ± 0.42
	4000	7.42 ± 0.66	7.12 ± 0.38	7.44 ± 0.69	7.47 ± 0.46
18:3n-3	400	2.38 ± 0.26	2.42 ± 0.42	2.65 ± 0.17	2.58 ± 0.40
	1000	2.32 ± 0.26	2.42 ± 0.30	2.48 ± 0.59	2.66 ± 0.11
	1500	2.27 ± 0.32	2.36 ± 0.59	2.44 ± 0.23	2.40 ± 0.29
	2000	2.33 ± 0.13	2.30 ± 0.32	2.34 ± 0.39	2.66 ± 0.38
	4000	2.27 ± 0.17	2.32 ± 0.05	2.30 ± 0.52	2.60 ± 0.39
18:4n-3	400	0.18 ± 0.21	0.22 ± 0.26	0.40 ± 0.08	0.32 ± 0.22
	1000	0.17 ± 0.23	0.35 ± 0.24	0.42 ± 0.13	0.28 ± 0.08
	1500	0.12 ± 0.25	0.28 ± 0.22	0.26 ± 0.17	0.39 ± 0.20
	2000	0.25 ± 0.21	0.42 ± 0.08	0.28 ± 0.18	0.46 ± 0.18
	4000	0.25 ± 0.21	0.30 ± 0.14	0.30 ± 0.19	0.40 ± 0.08
20:4n-6	400	2.62 ± 0.36	2.83 ± 0.51	2.17 ± 0.17	2.72 ± 1.14
	1000	2.67 ± 0.64	2.15 ± 0.79	2.45 ± 0.54	2.74 ± 0.45
	1500	3.17 ± 0.68	2.94 ± 0.56	2.68 ± 0.79	2.47 ± 0.87
	2000	2.80 ± 0.42	1.90 ± 0.43	2.62 ± 0.64	2.16 ± 0.30
	4000	2.73 ± 0.56	2.95 ± 0.74	2.62 ± 0.49	2.18 ± 0.38
20:5n-3	400	13.90 ± 2.59	13.15 ± 1.74	10.65 ± 1.36	12.40 ± 3.32
	1000	13.93 ± 3.85	11.68 ± 1.93	12.18 ± 1.23	13.06 ± 1.63
	1500	13.97 ± 2.28	14.50 ± 3.08	12.44 ± 1.86	12.20 ± 2.39
	2000	13.75 ± 2.20	10.44 ± 0.26	12.70 ± 2.68	11.00 ± 0.91
	4000	12.53 ± 1.10	14.40 ± 1.79	12.06 ± 2.70	10.55 ± 0.66
22:6n-3	400	19.07 ± 2.74	20.95 ± 3.96	14.82 ± 0.62	18.74 ± 6.66
	1000	20.33 ± 5.87	15.90 ± 3.94	16.43 ± 1.89	19.40 ± 2.67
	1500	20.80 ± 4.85	21.26 ± 4.51	18.28 ± 4.10	17.24 ± 5.61
	2000	20.23 ± 4.57	14.16 ± 2.34	18.22 ± 4.38	15.04 ± 1.56
	4000	17.88 ± 2.42	20.82 ± 4.36	17.58 ± 4.92	14.05 ± 1.81

Appendix XV

Mean chain length (MCL), ratios of polyunsaturated to saturated fatty acids (PUFA/SFA), ratios of 22:6n-3/20:4n-6 (DHA/ARA), and ratios of 18:3n-3/18:2n-6 (ALA/LA) in *Euphausia superba* sampled in experimental week's 1 – 5 (Jan – Feb; summer) of the one-year ocean acidification experiment. No significant differences were found between the control treatment (400 µatm pCO_2) and any of the elevated pCO_2 treatments (1000 – 4000 µatm pCO_2) during weeks 1, 2, 4 or 5 ($p > 0.05$). Units are mean ± SD. For each pCO_2 treatment $n = 3 – 7$.

	pCO_2	MCL	PUFA/SFA	DHA/ARA	ALA/LA
Week 1	400	18.75 ± 0.17	2.27 ± 0.26	7.31 ± 0.92	0.32 ± 0.04
	1000	18.77 ± 0.15	2.41 ± 0.66	7.57 ± 0.56	0.32 ± 0.02
	1500	18.82 ± 0.30	2.48 ± 0.58	6.56 ± 0.78	0.30 ± 0.03
	2000	18.77 ± 0.31	2.40 ± 0.47	7.20 ± 0.86	0.33 ± 0.01
	4000	18.68 ± 0.17	2.15 ± 0.29	6.63 ± 0.54	0.30 ± 0.01
Week 2	400	18.85 ± 0.24	2.69 ± 0.54	7.41 ± 0.23	0.33 ± 0.04
	1000	18.50 ± 0.37	1.96 ± 0.61	7.63 ± 0.98	0.34 ± 0.02
	1500	18.88 ± 0.29	2.71 ± 0.55	7.23 ± 0.60	0.32 ± 0.05
	2000	18.32 ± 0.22	1.67 ± 0.35	7.56 ± 0.68	0.32 ± 0.03
	4000	18.85 ± 0.31	2.80 ± 0.84	7.17 ± 0.86	0.32 ± 0.02
Week 4	400	18.45 ± 0.06	1.88 ± 0.12	6.86 ± 0.71	0.33 ± 0.03
	1000	18.52 ± 0.15	1.90 ± 0.26	6.84 ± 0.92	0.35 ± 0.06
	1500	18.64 ± 0.24	2.24 ± 0.48	6.94 ± 0.65	0.32 ± 0.03
	2000	18.60 ± 0.33	2.11 ± 0.46	6.96 ± 0.35	0.32 ± 0.03
	4000	18.56 ± 0.34	2.01 ± 0.55	6.63 ± 0.67	0.31 ± 0.04
Week 5	400	18.60 ± 0.44	2.26 ± 0.87	7.12 ± 0.94	0.35 ± 0.01
	1000	18.72 ± 0.19	2.37 ± 0.39	7.11 ± 0.43	0.36 ± 0.01
	1500	18.54 ± 0.37	2.06 ± 0.63	7.03 ± 0.39	0.32 ± 0.03
	2000	18.38 ± 0.11	1.78 ± 0.15	7.02 ± 0.75	0.35 ± 0.05
	4000	18.30 ± 0.14	1.64 ± 0.24	6.50 ± 0.37	0.34 ± 0.03

References

Ab Lah R, Kelaher BP, Bucher D, Benkendorff K (2018). Ocean warming and acidification affect the nutritional quality of the commercially-harvested turbinid snail *Turbo militaris*. *Marine Environmental Research* **141**: 100 – 108.

Adarme-Vega TC, Thomas-Hall SR, Schenk PM (2014). Towards sustainable sources for omega-3 fatty acids production. *Current Opinion in Biotechnology* **26**: 14 – 18.

Alberti G & Kils U (1983). Light- and electron microscopical studies on the anatomy and function of the gills of krill (Euphausiacea, Crustacea). *Polar Biology* **1**: 233 – 242.

Alonzo F, Nicol S, Virtue P, Nichols PD (2003). Lipids as trophic markers in Antarctic krill, I. Validation under controlled laboratory conditions. In: AHL Huiskes, WWC Gieskes, J Rozema, RML Schorno, SM van der Vies, WJ Wolff (eds) *Antarctic biology in a global context*. Backhuys Publishers, Leiden: 121 – 128.

Alonzo SH, Switzer PV, Mangel MM (2003). An ecosystem-based approach to management: using individual behaviour to predict the indirect effects of Antarctic krill fisheries on penguin foraging. *Journal of Applied Ecology* **40**: 692 – 702.

Alonzo F, Virtue P, Nicol S, Nichols PD (2005a) Lipids as trophic markers in Antarctic krill. II. Lipid composition of the body and digestive gland of *Euphausia superba* in controlled conditions. *Marine Ecology Progress Series* **296**: 65 – 79.

Alonzo F, Virtue P, Nicol S, Nichols PD (2005b). Lipids as trophic markers in Antarctic krill. III. Temporal changes in digestive gland lipid composition of *Euphausia superba* in controlled conditions. *Marine Ecology Progress Series* **296**: 81 – 91.

Andersson AJ, Mackenzie FT, Bates NR (2008). Life on the margin: implications of ocean acidification on Mg-calcite, high latitude and cold-water marine calcifiers. *Marine Ecology Progress Series* **373**: 265 – 273.

Aronson RB, Thatje S, McClintock JB, Hughes KA (2011). Anthropogenic impacts on marine ecosystems in Antarctica. *Annals of the New York Academy of Sciences* **1223**: 82 – 107.

Atkinson A, Hill SL, Pakhomov EA, Siegel V, Anadon R, Chiba S, Daly KL, Downie R, Fielding S, Fretwell P, Gerrish L, Hosie GW, Jessopp MJ, Kawaguchi S, Krafft BA, Loeb V, Nishikawa J, Peat HJ, Reiss CS, Ross RM, Quetin LB, Schmidt K, Steinberg DK, Subramaniam RC, Tarling GA,Ward P (2017). KRILLBASE: a circumpolar database of Antarctic krill and salp numerical densities, 1926–2016. *Earth System Science Data* **9** (1): 193 – 210.

Atkinson A, Meyer B, Stübing D, Hagen W, Schmidt K Bathmann UV (2002). Feeding and energy budgets of Antarctic krill *Euphausia superba* at the onset of winter - II. Juveniles and adults. *Limnology and Oceanography* **47**: 953 – 966.

Atkinson A, Nicol S, Kawaguchi S, Pakhomov EA, Quetin L, Ross R, Hill S, Reiss C, Seigel V, Tarling G (2012). Fitting *Euphausia superba* into southern ocean food-web models: A review of data sources and their limitations. *CCAMLR Science* **19**: 219 – 245.

Atkinson A, Siegel V, Pakhomov EA, Jessopp MJ, Loeb V (2009). A re-appraisal of the total biomass and annual production of Antarctic krill. *Deep Sea Research Part I:*

Oceanographic Research Papers **56** (5): 727 – 740.

Atkinson A, Siegel V, Pakhomov E, Rothery P (2004). Long-term decline in krill stock and increase in salps within the Southern Ocean. *Nature* **432** (7013): 100 – 103.

Atkinson A & Snyder R (1997). Krill-copepod interactions at South Georgia, Antarctica, I. omnivory by *Euphausia superba*. *Marine Ecology Progress Series* **160**: 63 – 76.

Auerswald L, Meyer B, Teschke M, Hagen W, Kawaguchi S (2015) Physiological response of adult Antarctic krill, *Euphausia superba*, to long-term starvation. *Polar Biology* **38**: 763 – 780.

Baker A, de C Boden BP, Brinton E (1990). *A practical guide to the Euphausiids of the world*. Natural History Museum Publications, London: 96 pp.

Barrett SM, Volkman JK, Dunstan GA, Leroi J-M (1995) Sterols in 14 species of marine diatoms (Bacillariophyta). *Journal of Phycology* **31**: 360 – 369.

Bell MV, Dick JR, Anderson TR, Pond DW (2007) Application of liposome and stable isotope tracer techniques to study polyunsaturated fatty acid biosynthesis in marine zooplankton. *Journal of Plankton Research* **29**: 417 – 422.

Bennett H, Bell JJ, Davy SK, Webster NS, & Francis DS (2018). Elucidating the sponge stress response; lipids and fatty acids can facilitate survival under future climate scenarios. *Global Change Biology*. doi: 10.1111/gcb.14116.

Bergé J-P & Barnathan G (2005). Fatty acids from lipids of marine organisms: molecular biodiversity, roles as biomarkers, biologically active compounds, and economical aspects. *Advances in Biochemical Engineering/Biotechnology* **96**: 49 – 125.

Bermúdez R, Feng Y, Roleda MY, Tatters AO, Hutchins DA, Larsen T, Boyd PW, Hurd CL, Riebessell U, Winder M (2015). Long-term conditioning to elevated pCO_2 and warming influences the fatty and amino acid composition of the diatom *Cylindrotheca fusiformis*. *PLOS ONE* **10** (5): e0123945. doi: 10.1371/journal.pone.0123945

Bermúdez R, Winder M, Stuhr A, Almén A-K, Engström-Öst J, Riebesell U (2016). Effect of ocean acidification on the structure and fatty acid composition of a natural plankton community in the Baltic Sea. *Biogeosciences* **13**: 6625 – 6635.

Bernard KS, Gunther LA, Mahaffey SH, Qualls KM, Sugla M, Saenz BT, Cossio AM, Walsh J, Reiss CS (2018). The contribution of ice algae to the winter energy budget of juvenile Antarctic krill in years with contrasting sea ice conditions. *ICES Journal of Marine Science*. doi: 10.1093/icesjms/fsy145.

Bi R, Ismar SMH, Sommer U, Zhao M (2018). Simultaneous shifts in elemental stoichiometry and fatty acids of *Emiliana huxleyi* in response to environmental changes. *Biogeosciences* **15**: 1029 – 1045.

Bligh EG & Dyer WJ (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37**: 911 – 917.

Bottino NR (1974). The fatty acids of Antarctic phytoplankton and Euphausiids. Fatty acid exchange among trophic levels of the Ross Sea. *Marine Biology* **27**: 197 – 204.

Boyd PW, Lennartz ST, Glover DM, Doney SC (2014). Biological ramifications of climate-change-mediated oceanic multi-stressors. *Nature Climate Change* **5**: 71 – 79.

Branch TA, Matsuoka K, Miyashita T (2006). Evidence for increases in Antarctic blue whales based on bayesian modelling. *Marine Mammal Science* **20** (4): 726 – 754.

Brewer PG (2009). A changing ocean seen with clarity. *Proceedings of the National Academy of Sciences of the United States of America* **106** (30): 12213 – 12214.

Brown M (2010). *Growth, Maturation and Physiology of Antarctic Krill*. PhD Thesis. Institute of Antarctic and Southern Ocean Studies, University of Tasmania.

Brown NEM, Bernhardt JR, Anderson KM, Harley CDG (2018). Increased food supply mitigates ocean acidification effects on calcification but exacerbates effects on growth. *Scientific Reports* **8**: 9800. doi: 10.1038/s41598-018-28012-w.

Brown M, Kawaguchi S, Candy S, Virtue P (2010). Temperature effects on the growth and maturation of Antarctic krill (*Euphausia superba*). *Deep Sea Research Part II: Topical Studies in Oceanography* **57** (7–8): 672 – 682.

Brown M, Kawaguchi S, Candy SG, Yoshida T, Virtue P, Nicol S (2013). Long-term effect of photoperiod, temperature and feeding regimes on the respiration rates of Antarctic krill (*Euphausia superba*). *Open Journal of Marine Science* **3**: 40 – 51.

Busch DS & McElhany P (2016). Estimates of the direct effect of seawater pH on the survival rate of species groups in the California Current ecosystem. *PLOS ONE* **11** (8): 1 – 28.

Butterworth DS & Thomson RB (1995). Possible effects of different levels of krill fishing on predators - some initial modelling attempts. *CCAMLR Science* **2**: 79 – 97.

Caldeira K & Wickett ME (2003). Anthropogenic carbon and ocean pH. *Nature* **425**: 365.

Calder PC (2010). Omega-3 fatty acids and inflammatory processes. *Nutrients* **2**: 355 – 374.

Calder PC & Grimble RF (2002). Polyunsaturated fatty acids, inflammatiuon and immunity. *European Journal of Clinical Nutrition* **56** (3): S14 – S19.

Carter HA, Ceballos-Osuna L, Miller NA, Stillman JH (2013). Impact of ocean acidification on metabolism and energetics during early life stages of the intertidal porcelain crab *Petrolisthes cinctipes*. *The Journal of Experimental Biology* **216**: 1412 – 1422.

CCAMLR (2015). Krill Fishery Report 2015. *CCAMLR, Hobart, Australia*. https://www.ccamlr.org/en/system/files/00%20KRI48%202015_0.pdf.

CCAMLR (2016). Krill Fishery Report 2016. *CCAMLR, Hobart, Australia.* https://www.ccamlr.org/en/system/files/00 KRI48 2016 v1_1.pdf.

CCAMLR (2017). Krill Fishery Report 2017. *CCAMLR, Hobart, Australia*. https://www.ccamlr.org/en/system/files/00 KRI48 2017.pdf.

Ciais P, Sabine C, Bala G, Bopp L, Brovkin V, Canadell J, Chhabra A, DeFries R, Galloway J, Heimann M, Jones C, Le Quéré C, Myneni RB, Piao S, Thornton P (2013). Carbon and Other Biogeochemical Cycles. In: TF Stocker, D Qin, G-K Plattner, M Tignor, SK Allen, J Boschung, A Nauels, Y Xia, V Bex, PM Midgley (eds) *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge University Press, Cambridge, United Kingdom: 465 – 570.

Claphan PJ, Young SB, Brownell Jr RL (1999). Baleen whales: conservation issues and the status of the most endangered populations. *Mammal Review* **29**: 35 – 60.

Clarke A (1980). The biochemical composition of krill, *Euphausia superba* Dana, from South Georgia. *Journal of Experimental Marine Biology and Ecology* **43**: 221 – 236.

Clarke A (1984) Lipid content and composition of Antarctic krill, *Euphausia superba* Dana. *Journal of Crustacean Biology* **4**: 285 – 294.

Clarke A (1988). Seasonality in the Antarctic marine environment. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry* **90** (3): 461 – 473.

Clarke A & Tyler PA (2008). Adult Antarctic krill feeding at abyssal depths. *Current Biology* **18** (4): 282 – 285.

Coll M & Guershon M (2002). Omnivory in terrestrial arthropods: mixing plant and prey diets. *Annual Review of Entomology* **47**: 267 – 297.

Constable AJ, Costa DP, Schofield O, Newman L, Urban ER, Fulton EA, Melbourne-Thomas J, Ballerini T, Boyd PW, Brandt A, de la Mare WK, Edwards M, Eléaume M, Emmerson L, Fennel K, Fielding S, Griffiths H, Gutt J, Hindell MA, Hofmann EE, Jennings S, La HS, McCurdy A, Mitchell BG, Moltmann T, Muelbert M, Murphy E, Press AJ, Raymond B, Reid K, Reiss C, Rice J, Salter I, Smith DC, Song S, Southwell C, Swadling KM, de Putte AV, Willis Z. (2016). Developing priority variables ("ecosystem Essential Ocean Variables" — eEOVs) for observing dynamics and change in Southern Ocean ecosystems. *Journal of Marine Systems* **161**: 26 – 41.

Constable A, de la Mare WK, Agnew D, Everson I, Miller D (2000). Managing fisheries to conserve the Antarctic marine ecosystem: practical implementation of the Convention on the Conservation of Antarctic Marine Living Resources (CCAMLR). *ICES Journal of Marine Science* **57**: 778 – 791.

Constable AJ & Kawaguchi S (2018). Modelling growth and reproduction of Antarctic krill, *Euphausia superba*, based on temperature, food and resource allocation amongst life history functions. *ICES Journal of Marine Science* **75** (2): 738 – 750.

Cooper HL, Potts DC, Paytan A (2016). Metabolic responses of the North Pacific krill, *Euphausia pacifica*, to short- and long-term pCO_2 exposure. *Marine Biology* **163**: 207.

Cooper HL, Potts DC, Paytan A (2017). Effects of elevated pCO_2 on the survival, growth, and moulting of the Pacific krill species, *Euphausia pacifica*. *ICES Journal of Marine Sci*ence **74**: 1005 – 1012.

Corsolini S & Borghesi N (2017). A comparative assessment of fatty acids in Antarctic organisms from the Ross Sea: Occurence and distribution. *Chemosphere* **174**: 747 – 753.

Cox MJ, Candy S, de la Mare WK, Nicol S, Kawaguchi S, Gales N (2018). No evidence for a decline in the density of Antarctic krill *Euphausia superba* Dana, 1850, in the Southwest Atlantic sector between 1976 and 2016. *Journal of Crustacean Biology* **38** (6): 656 – 661.

Cripps GC & Atkinson A (2000). Fatty acid composition as an indicator of carnivory in Antarctic krill, *Euphausia superba*. *Canadian Journal of Fisheries and Aquatic Sciences* **57**: 31 – 37.

Cripps GC & Hill HJ (1998). Changes in lipid composition of copepods and *Euphausia superba* associated with diet and environmental conditions in the marginal ice zone, Bellingshausen Sea, Antarctica. *Deep-Sea Research Part I Oceanographic Research Papers* **45**: 1357 – 1381.

Cripps GC, Watkins JL, Hill HJ, Atkinson A. (1999). Fatty acid content of Antarctic krill *Euphausia superba* at South Georgia related to regional populations and variations in diet. *Marine Ecology Progress Series* **181**: 177 – 188.

Croxall JP, Reid K, Prince PA (1999). Diet, provisioning and productivity responses of marine predators to differences in availability of Antarctic krill. *Marine Ecology Progress Series*, **177**: 115 – 131.

Cuzin-Roudy J & Amsler MO (1991). Ovarian development and sexual maturity staging in Antarctic krill *Euphausia superba* Dana (Euphausiacea). *Journal of Crustacean Biology* **11**: 236 – 249.

Cuzin-Roudy J, Irisson J-O, Penot F, Kawaguchi S, & Vallet C (2014). Southern Ocean Euphausiids. In: C De Broyer, P Koubbi, HJ Griffiths, B Raymond, C Udekem d'Acoz, AP Van de Putte, B Danis, B David, S Grant, J Gutt, C Held, G Hosie, F Huettmann, A Post, Y. Ropert-Coudert (eds) *Biogeographic atlas of the Southern Ocean*. Scientific Comittee on Antarctic Research, Cambridge: 309 – 320.

Dalsgaard J, St John M, Kattner G, Muller-Navarra D, Hagen W (2003). Fatty acid trophic markers in the pelagic marine environment. *Advances in Marine Biology* **46**: 225 – 340.

Daly KL (1990). Overwintering development, growth, and feeding of larval *Euphausia superba* in the Antarctic marginal ice zone. *Limnology and Oceanography* **35** (7): 1564 – 1576.

Dana JD (1850). Synopsis generum crustaceorum ordinis "Schizopoda." *American Journal of Science and Arts, ser. 2*, **9**: 129 – 133.

Dawson AL, Kawaguchi S, King CK, Townsend KA, King R, Huston WM, Bengtson Nash SM (2018). Turning microplastics into nanoplastics through digestive fragmentation by Antarctic krill. *Nature Communications* **9**: 1001. doi: 10.1038/s41467-018-03465-9.

De Troch M, Boeckx P, Cnudde C, Van Gansbeke D, Vanreusel A, Vincx M, Caramujo MJ (2012). Bioconversion of fatty acids at the basis of marine food webs: insights from a compound-specific stable isotope analysis. *Marine Ecology Progress Series* **465**: 53 – 67.

Deppeler SL & Davidson AT (2017). Southern Ocean phytoplankton in a changing climate. *Frontiers in Marine Science* **4** (40): 1 – 28.

Descamps S, Tarroux A, Cherel Y, Delord K, Godo OR, Kato A, Krafft BA, Lorentsen S-H, Ropert-Coudert Y, Skaret G, Varpe Ø (2016). At-sea distribution and prey selection of Antarctic petrels and commercial krill fisheries. *PLOS ONE* **11** (8): e0156968. doi: 10.1371/journal.pone.0156968.

Díaz-Gil C, Catalán IA, Palmer M, Faulk CK, Fuiman LA (2015). Ocean acidification increases fatty acids levels of larval fish. *Biology Letters* **11** (7): 20150331. doi: 10.1098/rsbl.2015.0331.

Dickson AG, Sabine CL, Christian JR (2007). *Guide to Best Practices for Ocean CO_2 Measurements*. PICES Special Publication 3, North Pacific Marine Science Organization: 191 pp.

Doney SC, Fabry VJ, Feely RA, Kleypas JA (2009). Ocean acidification: the other CO_2 problem. *Annual Review of Marine Science* **1**: 169 – 192. doi: 10.1146/annurev.marine.010908.163834.

Doney SC & Schimel DS (2007). Carbon and climate system coupling on timescales from the precambrian to the anthropocene. *Annual Review of Environment and Resources* **32**: 31 – 66.

Ekwurzel B, Boneham J, Dalton MW, Heede R, Mera RJ, Allen MR, Frumhoff PC (2017). The rise in global atmospheric CO_2, surface temperature, and sea level from emissions traced to major carbon producers. *Climatic Change* **144** (4): 579 – 590.

Ericson JA, Hellessey N, Nichols PD, Kawaguchi S, Nicol S, Hoem N, Virtue P (2018a). Seasonal and interannual variations in the fatty acid composition of adult *Euphausia superba* Dana, 1850 (Euphausiacea) samples derived from the Scotia Sea krill fishery. *Journal of Crustacean Biology* **38** (6): 656 – 661.

Ericson JA, Hellessey N, Kawaguchi S, Nicol S, Nichols P, Hoem N, Virtue P (2018b). Adult Antarctic krill proves resilient in a simulated high CO_2 ocean. *Communications Biology* **1:** 190. doi: 10.1038/s42003-018-0195-3.

Ernst R, Ejsing CS, Antonny B (2016). Homeoviscous adaptation and the regulation of membrane lipids. *Journal of Molecular Biology* **428**: 4776 – 4791.

Fabry VJ, McClintock JB, Mathis JT, Grebmeier JM (2009). Ocean acidification at high latitudes: the bellweather. *Oceanography* **22** (4): 160 – 171.

Falk-Petersen S, Hagen W, Kattner G, Clarke A, Sargent J (2000). Lipids, trophic relationships, and biodiversity in Arctic and Antarctic krill. *Canadian Journal of Fisheries and Aquatic Sciences* **57** (3): 178 – 191.

Fay G, Link JS, Hare JA (2017). Assessing the effects of ocean acidification in the Northeast US using an end-to-end marine ecosystem model. *Ecological Modelling* **347**: 1 – 10.

Feely RA, Doney SC, Cooley SR (2009). Ocean acidification: present conditions and future changes in a high-CO_2 world. *Oceanography* **22** (4): 36 – 47.

Feijão E, Gameiro C, Franzitta M, Duarte B, Caçador I, Cabrita MT, Matos AR (2017). Heat wave impacts on the model diatom *Phaeodactylum tricornutum*: Searching for photochemical and fatty acid biomarkers of thermal stress. *Ecological Indicators* **95** (2): 1026 – 1037.

Fennel W & Neumann T (2015). *Introduction to the modelling of marine ecosystems* (2nd Edition). Elsevier, Amsterdam, Netherlands.

Flores H, Atkinson A, Kawaguchi S, Krafft BA, Milinevsky G, Nicol S, Reiss C, Tarling GA,Werner R, Bravo Rebolledo E, Cirelli V, Cuzin-Roudy J, Fielding S, Groeneveld JJ, Haraldsson M, Lombana A, Marschoff E, Meyer B, Pakhomov EA, Rombolá E, Schmidt K, Siegel V, Teschke M, Tonkes H, Toullec JY, Trathan PN, Tremblay N, Van de Putte AP, van Franeker JA, Werner, T (2012). Impact of climate change on Antarctic krill. *Marine Ecology Progress Series* **458**: 1 – 19.

Foo SA & Byrne M (2016). Acclimatization and adaptive capacity of marine species in a changing ocean. *Advances in Marine Biology* **74**: 69 – 116.

Fox CJJ (1907). On the coefficients of absorption of the atmospheric gases in distilled water and seawater. Part 1: Nitrogen and Oxygen. *ICES Journal of Marine Sci*ence **s1** (41): 3 – 23.

Freeman NM, Lovenduski NS, Gent PR (2016). Temporal variability in the Antarctic Polar

Front (2002–2014). *Journal of Geophysical Research: Oceans* **121**: 7263 – 7276.

Fricke H, Gercken G, Schreiber W, Oehlenschläger J (1984). Lipid, sterol and fatty acid composition of Antarctic krill (*Euphausia superba* Dana). *Lipids* **19**: 821 – 827.

Gao Y, Zheng S-C, Zheng C-Q, Shi Y-C, Xie X-L, Wang K-J, Liu HP (2018). The immune-related fatty acids are responsive to CO_2 driven seawater acidification in a crustacean brine shrimp *Artemia sinica*. *Developmental and Comparative Immunology* **81**: 342 – 347.

Garzke J, Hansen T, Ismar SMH, Sommer U (2016). Combined effects of ocean warming and acidification on copepod abundance, body size and fatty acid content. *PLOS ONE* **11** (5): e0155952. doi:10.1371/journal.pone.0155952.

Gattuso JP, Magnan A, Bille R, Cheung WW, Howes EL, Joos F, Allemand D, Bopp L, Cooley SR, Eakin CM, Hoegh-Guldberg O, Kelly RP, Pörtner H-O, Rogers AD, Baxter JM, Laffoley D, Osborn D, Rankovic A, Rochette J, Sumaila UR, Treyer S, Turley C (2015). Contrasting futures for ocean and society from different anthropogenic CO_2 emissions scenarios. *Science* **349** (6243): aac4722. doi: 10.1126/science.aac4722.

Gigliotti JC, Davenport MP, Beamer SK, Tou JC, Jaczynski J (2011). Extraction and characterisation of lipids from Antarctic krill (*Euphausia superba*). *Food Chemistry* **125**: 1028 – 1036.

Graeve M, Hagen W, Kattner G (1994) Herbivorous or omnivorous? On the significance of lipid compositions as trophic markers in Antarctic copepods. *Deep Sea Research Part I : Oceanographic Research Papers* **41**: 915 – 924.

Griffith GP, Fulton EA, Gorton R, Richardson AJ (2012). Predicting interactions among fishing, ocean warming, and ocean acidification in a marine ecosystem with whole-ecosystem models. *Conservation Biology* **26** (6): 1145 – 1152.

Hagen W, Kattner G, Graeve M (1993) *Calanoides acutus* and *Calanus propinquus*, Antarctic copepods with different lipid storage modes via wax esters or triacylglycerols. *Marine Ecology Progress Series* **97**: 135 – 142.

Hagen W, Kattner G, Terbruggen A, Van Vleet ES (2001). Lipid metabolism of the Antarctic krill *Euphausia superba* and its ecological implications. *Marine Biology* **139**: 95 – 104.

Hagen W & Schnack-Schiel SB (1996). Seasonal lipid dynamics in dominant Antarctic copepods: Energy for overwintering or reproduction? *Deep Sea Research Part I: Oceanographic Research Papers* **43** (2): 139 – 158.

Hagen W, VanVleet ES, Kattner G (1996). Seasonal lipid storage as overwintering strategy of Antarctic krill. *Marine Ecology Progress Series* **134**: 85 – 89.

Hagen W, Yoshida T, Virtue P, Kawaguchi S, Swadling KM, Nicol S, Nichols PD (2007). Effect of a carnivorous diet on the lipids, fatty acids and condition of Antarctic krill, *Euphausia superba*. *Antarctic Science* **19**: 183 – 188.

Harvey BP, Gwynn-Jones D, Moore PJ (2013). Meta-analysis reveals complex marine biological responses to the interactive effects of ocean acidification and warming. *Ecology and Evolution* **3** (4): 1016 – 1030.

Hauri C, Friedrich T & Timmermann A (2016). Abrupt onset and prolongation of aragonite undersaturation events in the Southern Ocean. *Nature Climate Change* **6**: 172 – 176.

Hazel JR (1995). Thermal adaptation in biological membranes: is homeoviscous adaptation the

explanation? *Annual Review of Physiology* **57**: 19 – 42.

Hellessey N, Ericson JA, Nichols PD, Kawaguchi S, Nicol S, Hoem N, Virtue P (2018). Seasonal and interannual variation in the lipid content and composition of *Euphausia superba* Dana 1850 (Euphausiacea) samples derived from the Scotia Sea fishery. *Journal of Crustacean Biology* **38** (6): 673 – 681.

Hendriks IE, Duarte CM, Álvarez M. (2010). Vulnerability of marine biodiversity to ocean acidification: A meta-analysis. *Estuarine, Coastal and Shelf Science* **86** (2): 157 – 164.

Henry RP & Wheatly MG (1992). Interaction of respiration, ion regulation, and acid-base balance in the everyday life of aquatic crustaceans. *American Zoologist* **32**: 407 – 416.

Hernando M, Schloss IR, Almandoz GO, Malanga G, Varela DE, De Troch M (2018). Combined effects of temperature and salinity on fatty acid content and lipid damage in Antarctic phytoplankton. *Journal of Experimental Marine Biology and Ecology* **503**: 120 – 128.

Hill SL, Murphy EJ, Reid K, Trathan PN, Constable AJ (2006). Modelling Southern Ocean ecosystems: krill, the food-web, and the impacts of harvesting. *Biological Reviews* **81** (4): 581 – 608.

Hixson SM & Arts MT (2016). Climate warming is predicted to reduce omega-3, long-chain, polunsaturated fatty acid production in phytoplankton. *Global Change Biology* **22**: 2744 – 2755.

Hoegh-Guldberg O & Bruno JF (2010). The impact of climate change on the world's marine ecosystems. *Science* **328** (5985): 1523 – 1528.

Höring F, Teschke M, Suberg L, Kawaguchi SS, Meyer B (2018). Light regime affects the seasonal cycle of Antarctic krill: Impacts on growth, feeding, lipid metabolism and maturity. *Canadian Journal of Zoology* **96** (11): 1203 – 1213.

Hurd CL, Lenton A, Tilbrook B, Boyd PW (2018). Current understanding and challenges for oceans in a higher-CO_2 world. *Nature Climate Change* **8** (8): 686 – 694.

IPCC (2014). Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, R.K. Pachauri and L.A. Meyer (eds)]. IPCC, Geneva, Switzerland: 151 pp.

Iverson SJ, Field C, Don Bowen W, Blanchard W (2004). Quantitative fatty acid signature analysis: A new method of estimating predator diets. *Ecological Monographs* **74** (2): 211 – 235.

Jager T, Martin BT, Zimmer EI (2013). DEBkiss or the quest for the simplest generic model of animal life history. *Journal of Theoretical Biology* **328**: 9 – 18.

Jager T & Ravagnan E (2015). Parameterising a generic model for the dynamic energy budget of Antarctic krill *Euphausia superba*. *Marine Ecology Progress Series* **519**: 115 – 128.

Ju S-J & Harvey HR (2004). Lipids as markers of nutritional condition and diet in the Antarctic krill *Euphausia superba* and *Euphausia crystallorophias* during austral winter. *Deep-Sea Research Part II: Topical Studies in Oceanography* **51**: 2199 – 2214.

Kanazawa A (2001). Sterols in marine invertebrates. *Fisheries Science* **67**: 997 – 1007.

Kattner G & Hagen W (1995). Polar herbivorous copepods - different pathways in lipid biosynthesis. *ICES Journal of Marine Science* **52** (3–4): 329 – 335.

Kattner G & Hagen W (1998) Lipid metabolism of the Antarctic euphausiid *Euphausia crystallorophias* and its ecological implications. *Marine Ecology Progress Series* **170**: 203 – 213.

Kattner G, Hagen W, Lee RF, Campbell R, Deibel D, Falk-Petersen S, Graeve M, Hansen BW, Hirche HJ, Jónasdóttir SH, Madsen ML, Mayzaud P, Müller-Navarra D, Nichols PD, Paffenhöfer G-A, Pond D, Saito H, Stübing D, Virtue P (2007). Perspectives on marine zooplankton lipids. *Canadian Journal of Fisheries and Aquatic Sciences* **64** (11): 1628 – 1639.

Kawaguchi S (2016). Reproduction and larval development in Antarctic krill (*Euphausia superba*). In: V Siegel (ed) *Biology and Ecology of Antarctic Krill (Advances in Polar Ecology)*. Springer International Publishing, Switzerland: 225 – 246.

Kawaguchi S, Ishida A, King R, Raymond B, Waller N, Constable A, Nicol S, Wakita M, Ishimatsu A (2013). Risk maps for Antarctic krill under projected Southern Ocean acidification. *Nature Climate Change* **3** (9): 843 – 847.

Kawaguchi S, King R, Meijers R, Osborn JE, Swadling KM, Ritz DA, Nicol S (2010). An experimental aquarium for observing the schooling behaviour of Antarctic krill (*Euphausia superba*). *Deep Sea Research Part II: Topical Studies in Oceanography* **57** (7 – 8): 683 – 692.

Kawaguchi S, Kurihara H, King R, Hale L, Berli T, Robinson JP, Ishida A, Wakita M, Virtue P, Nicol S, Ishimatsu A (2011). Will krill fare well under Southern Ocean acidification? *Biology Letters* **7**(2): 288 – 291.

Kawaguchi S & Nicol S (2007). Learning about Antarctic krill from the fishery. *Antarctic Science* **19** (2): 219 – 230.

Kawaguchi S, Nicol S, Press AJ (2009). Direct effects of climate change on the Antarctic krill fishery. *Fisheries Management and Ecology* **16**: 424 – 427.

Kawaguchi S, Toshihiro Y, Finley L, Cramp P, Nicol S (2007). The krill maturity cycle: a conceptual model of the seasonal cycle in Antarctic krill. *Polar Biology* **30**: 689 – 698.

Kilada R, Reiss CS, Kawaguchi S, King RA, Matsuda T, Ichii T (2017). Validation of band counts in eyestalks for the determination of age of Antarctic krill, *Euphausia superba*. *PLOS ONE* **12** (2): e0171773. doi:10.1371/journal.pone.0171773.

King R, Nicol S, Cramp P, Swadling KM (2003). Krill maintenance and experimentation at the australian antarctic division. *Marine and Freshwater Behaviour and Physiology* **36**: 271 – 283.

King AL, Jenkins BD, Wallace JR, Liu Y, Wikfors GH, Milke LM, Meseck SL (2015). Effects of CO_2 on growth rate, C:N:P, and fatty acid composition of seven marine phytoplankton species. *Marine Ecology Progress Series* **537**: 59 – 69.

Kirkwood JM (1984). *ANARE Research Notes 1. A guide to the Euphausiacea of the Southern Ocean.* Australian Antarctic Division, Kingston, Tasmania, Australia.

Kock K-H, Reid K, Croxall J, Nicol S (2007). Fisheries in the Southern Ocean: an ecosystem approach. *Philosophical Transactions of the Royal Society B* **362**: 2333 – 2349.

Kopczynska, EE (1992). Dominance of microflagellates over diatoms in the Antarctic areas of deep vertical mixing and krill concentrations. *Journal of Plankton Research* **14**: 1031 – 1054.

Krafft BA, Skaret G, Knutsen T (2015). An Antarctic krill (*Euphausia superba*) hotspot: population characteristics, abundance and vertical structure explored from a krill fishing vessel. *Polar Biology* **38**: 1687 – 1700.

Kratina P, LeCraw RM, Ingram T, Anholt BR (2012). Stability and persistence of food webs with omnivory: Is there a general pattern? *Ecosphere* **3** (6): 1 – 18.

Kroeker KJ, Kordas RL, Crim RN, Singh GG (2010). Meta-analysis reveals negative yet variable effects of ocean acidification on marine organisms. *Ecology Letters* **13** (11): 1419 – 1434.

Kurihara H & Ishimatsu A (2008). Effects of high CO_2 seawater on the copepod (*Acartia tsuensis*) through all life stages and subsequent generations. *Marine Pollution Bulletin* **56**: 1086 – 1090.

Kurihara H, Shimode S, Shirayama Y (2004a). Sub-lethal effects of elevated concentration of CO_2 on planktonic copepods and sea urchins. *Journal of Oceanography* **60**: 743 – 750.

Kurihara H, Shimode S, Shirayama Y (2004b). Effects of raised CO_2 concentration on the egg production rate and early development of two marine copepods (*Acartia steuri* amd *Acartia eythraea*). *Marine Pollution Bulletin* **49**: 721 – 727.

Landschützer P, Gruber N, Haumann FA, Rödenbeck C, Bakker DCE, van Heuven S, Hoppema M, Metzl N, Sweeney C, Takahashi T, Tilbrook B, Wanninkhof R (2015). The reinvigoration of the Southern Ocean carbon sink. *Science* **349** (6253): 1221 – 1224.

Li W & Gao K (2012) A marine secondary producer respires and feeds more in a high CO_2 ocean. *Marine Pollution Bulletin* **64**: 699 – 703.

Loeb V, Siegel V, Holm-Hansen O, Hewitt R, Fraser W, Trivelpiece W, Trivelpiece S (1997). Effects of sea-ice extent and krill or salp dominance on the Antarctic food web. *Nature* **387** (6636): 897 – 900.

Los DA & Murata N (2004). Membrane fluidity and its roles in the perception of environmental signals. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1666** (1): 142 – 157.

Lynnes AS, Reid K, Croxall JP (2004). Diet and reproductive success of Adelie and chinstrap penguins: linking response of predators to prey population dynamics. *Polar Biology* **27**: 544 – 554.

Malekar VC, Morton JD, Hider RN, Cruickshank RH, Hodge S, Metcalf VJ (2018). Effect of elevated temperature on membrane lipid saturation in Antarctic notothenioid fish. *PeerJ* **6**: e4765. doi:org/10.7717/peerj.4765.

Mangel M & Switzer PV (1998). A model at the level of the foraging trip for the indirect effects of krill (*Euphausia superba*) fisheries on krill predators. *Ecological Modelling* **105**: 235 – 256.

Matson PG, Yu PC, Sewell MA, Hofmann GE (2012). Development under elevated pCO_2 conditions does not affect lipid utilization and protein content in early life-history stages of the purple sea urchin, *Strongylocentrotus purpuratus*. *Biological Bulletin* **223** (3): 312 – 327.

Marr J (1962). The natural history and geography of the Antarctic krill (*Euphausia superba* Dana). *Discovery Reports* **32**: 33 – 464.

Marshall CT, Yaragina NA, Lambert Y, Kjesbu OS (1999). Total lipid energy as a proxy for total egg production by fish stocks. *Nature* **402**: 288 – 290.

Martin-Creuzburg D & von Elert E (2009) Ecological significance of sterols in aquatic food webs. In: MT Arts, MT Brett, M Kainz (eds) *Lipids in Aquatic Ecosystems*. Springer-Verlag, New York: 43 – 64.

Massom RA & Stammerjohn SE (2010). Antarctic sea ice change and variability – Physical and ecological implications. *Polar Science* **4** (2): 149 – 186.

Mayzaud P (1997) Spatial and life-cycle changes in lipid and fatty acid structure of the Antarctic euphausiid *Euphausia superba*. In: J Valencia, DWH Walton (eds) *Antarctic Communities: Species, Structure and Survival.* Cambridge University Press, Cambridge: 284 – 294.

Mayzaud P, Albessard E, Cuzin-Roudy J (1998) Changes in lipid compostion of the Antarctic krill *Euphausia superba* in the Indian sector of the Antarctic ocean: influence of geographical location, sexual maturity stage and distribution among organs. *Marine Ecology Progress Series* **173**: 149 – 162.

Mayzaud P, Albessard E, Virtue P, Boutoute M (2000) Environmental constraints on the lipid composition and metabolism of euphausiids: the case of *Euphausia superba* and *Meganyctiphanes norvegica*. *Canadian Journal of Fisheries and Aquatic Sciences* **57**: 91 – 103.

Mayzaud P, Virtue P, Albessard E (1999) Seasonal variations in the lipid and fatty acid composition of the euphausiid *Meganyctiphanes norvegica* from the Ligurian Sea. *Marine Ecology Progress Series* **186**: 199 – 210.

McNeil BI & Matear RJ (2008). Southern Ocean acidification: a tipping point at 450-ppm atmospheric CO_2. *Proceedings of the National Academy of Sciences of the United States of America* **105** (48): 18860 – 18864.

Melbourne-Thomas J, Constable A, Wotherspoon S, Raymond B (2013). Testing paradigms of ecosystem change under climate warming in Antarctica. *PLOS ONE* **8** (2): 1 – 9.

Melzner F, Gutowska MA, Langenbuch M, Dupont S, Lucassen M, Thorndyke MC, Bleich M, Pörtner, H-O (2009). Physiological basis for high CO_2 tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? *Biogeosciences* **6**: 2313 – 2331.

Meredith MP & King JC (2005). Rapid climate change in the ocean west of the Antarctic Peninsula during the second half of the 20th century. *Geophysical Research Letters* **32**: L19604. doi: 10.1029/2005GL024042.

Meyer B (2012). The overwintering of Antarctic krill, *Euphausia superba*, from an ecophysiogical perspective. *Polar Biology* **35**: 15 – 37.

Meyer B, Auerswald L, Siegel V, Spahić C, Pape C, Fach BA, Teschke M, Lopata AL, Fuentes V (2010). Seasonal variation in body composition, metabolic activity, feeding, and growth of adult krill *Euphausia superba* in the Lazarev Sea. *Marine Ecology Progress Series* **398**: 1 – 18.

Meyer B & Teschke M (2016). Physiology of *Euphausia superba*. In: V Siegel (ed) *Biology and Ecology of Antarctic Krill (Advances in Polar Ecology)*. Springer International Publishing, Switzerland: 145 – 174.

Monroig Ó & Kabeya N (2018). Desaturases and elongases involved in polyunsaturated fatty acid biosynthesis in aquatic invertebrates: a comprehensive review. *Fisheries Science* **84**: 911 – 928.

Monroig Ó, Tocher DR, Navarro JC (2013). Biosynthesis of polyunsaturated fatty acids in marine invertebrates: recent advances in molecular mechanisms. *Marine Drugs* **11**: 3998 – 4018.

Munro DR, Lovenduski NS, Takahashi T, Stephens BB, Newberger T, Sweeney C (2015). Recent evidence for a strengthening CO_2 sink in the Southern Ocean from carbonate system measurements in the Drake Passage (2002–2015). *Geophysical Research Letters* **42**: 7623 – 7630.

Murphy EJ (1995). Spatial structure of the Southern Ocean ecosystem: Predator-prey linkages in Southern Ocean food webs. *Journal of Animal Ecology* **64**: 333 – 347.

Murphy EJ, Morris DJ, Watkins JL, Priddle J (1988). Scales of interaction between Antarctic krill and the environment. In: D Sahrhage (ed) *Antarctic Ocean and Resources Variability*. Springer, Berlin, Heidelberg: 120 – 130.

Murphy EJ, Watkins JL, Trathan PN, Reid K, Meredith MP, Thorpe SE, Johnston NM, Clarke A, Tarling GA, Collins MA, Forcada J, Shreeve RS, Atkinson A, Korb R, Whitehouse MJ, Ward P, Rodhouse PG, Enderlein P, Hirst AG, Martin AR, Hill SL, Staniland IJ, Pond DW, Briggs DR, Cunningham NJ, Fleming AH (2007). Spatial and temporal operation of the Scotia Sea ecosystem: a review of large-scale links in a krill centred food web. *Philosophical Transactions of the Royal Society B - Biological Sciences* **362**: 113 – 148.

Murray CS, Fuimann LA, Baumann H (2017). Consequences of elevated CO_2 exposure across multiple life stages in a coastal forage fish. *ICES Journal of Marine Science* **74** (4): 1051 – 1061.

Nelson DL & Cox MM (2012). *Lehninger Principles of Biochemistry* (6th Edition). WH Freeman and Company, New York.

Nicol S (2018). *The Curious Life of Krill* (2nd Edition). Island Press, Washington DC.

Nicol S, Constable AJ, Pauly T (2000). Estimates of circumpolar abundance of Antarctic krill based on recent acoustic density measurements. *CCAMLR Science* **7**: 87 – 99.

Nicol S & Endo Y (1999). Krill fisheries: Development, management and ecosystem implications. *Aquatic Living Resources* **12** (2): 105 – 120.

Nicol S, Foster J, Kawaguchi S (2012). The fishery for Antarctic krill - recent developments. *Fish and Fisheries* **13** (1): 30 – 40. doi: 10.1111/j.1467-2979.2011.00406.x.

Nichols PD, Skerratt JH, Davidson A, Burton H, McMeekin TA (1991). Lipids of cultured *Phaeocystis pouchetii*: Signatures for food-web, biogeochemical and environmental studies in Antarctica and the Southern ocean. *Phytochemistry* **30**: 3209 – 3214.

O'Brien C, Virtue P, Kawaguchi S, Nichols PD (2011). Aspects of krill growth and condition during late winter-early spring off East Antarctica (110–130°E). *Deep-Sea Research Part II: Topical Studies in Oceanography* **58**: 1211 – 1221.

Olsen E, Kaplan IC, Ainsworth C, Fay G, Gaichas S, Gamble R, Girardin R, Eide CH, Ihde TF, Morzaria-Luna HN, Johnson KF, Savina-Rolland M, Townsend H, Weijerman M, Fulton EA, Link JS (2018). Ocean futures under ocean acidification, marine protection, and changing fishing pressures explored using a worldwide suite of ecosystem models. *Frontiers in Marine Science* **5**: 64. doi:10.3389/fmars.2018.00064.

Opstad I, Mangor-Jensen A, Sperfeld E, Semb Johansen I, Fransson A, Chierici M, Dalpadado P (2018). Effects of high pCO_2 in the northern krill *Thysanoessa inermis* in relation to carbonate chemistry of its collection area, Rijpfjorden. *Marine Biology* **165**: 116. doi:10.1007/s00227-018-3370-7.

Parrish CC (2013). Lipids in Marine Ecosystems. *ISRN Oceanography* 1 – 16. doi: 10.5402/2013/604045.

Parrish CC, Abrajano TA, Budge SM, Helleur RJ, Hudson ED, Pulchan K, Ramos C (2000) Lipid and phenolic biomarkers in marine ecosystems: analysis and applications. In: *The Handbook of Environmental Chemistry Vol. 5 Part D Marine Chemistry*. Springer-Verlag, Berlin, Heidelberg: 193 – 223.

Pethybridge HR, Choy CA, Polovina JJ, Fulton EA (2018). Improving marine ecosystem models with biochemical tracers. *Annual Review of Marine Science* **10** (1): 199 – 228.

Pethybridge HR, Parrish CC, Morrongiello J, Young JW, Farley JH, Gunasekera RM, & Nichols PD (2015). Spatial patterns and temperature predictions of tuna fatty acids: tracing essential nutrients and changes in primary producers. *PLOS ONE* **10** (7): 1 – 17.

Phleger CF, Nelson MM, Mooney BD, Nichols PD (2002) Interannual and between species comparison of the lipids, fatty acids and sterols of Antarctic krill from the US AMLR Elephant Island survey area. *Comparitive Biochemistry and Physiology Part B Biochem istry and Molecular Biology* **131**: 733 – 747.

Phleger CF, Nichols PD, Virtue P (1998). Lipids and trophodynamics of Antarctic zooplankton. *Comparative Biochemistry and Physiology Part B Biochemistry and Molecular Biology* **120**: 311 – 323.

Pierrot D, Lewis E, Wallace DWR (2006). MS Excel program developed for CO_2 system calculations. Carbon Dioxide Information Analysis Center, Oak Ridge: 10.3334/CDIAC/otg.CO2SYS_XLS_CDIAC105a.

Plagányi EE & Butterworth DS (2012). The Scotia Sea krill fishery and its possible impacts on dependent predators: modeling localized depletion of prey. *Ecological Applications* **22** (3): 748 – 761.

Pond DW, Atkinson A, Shreeve RS, Tarling G, Ward P (2005). Diatom fatty acid biomarkers indicate recent growth rates in Antarctic krill. *Limnology and Oceanography* **50**: 732 – 736.

Pond D, Watkins J, Priddle J, Sargent J (1995). Variation in the lipid content and composition of Antarctic krill *Euphausia superba* at South Georgia. *Marine Ecology Progress Series* **117**: 49 – 57.

Pörtner H-O, Karl DM, Boyd PW, Cheung WWL, Lluch-Cota SE, Nojiri Y, Schmidt DN, Zavialov PO (2014). Ocean Systems. In: CB Field, VR Barros, DJ Dokken, KJ Mach, MD Mastrandrea, TE Bilir, M Chatterjee, KL Ebi, YO Estrada, RC Genova, B Girma, ES Kissel, AN Levy, S MacCracken, PR Mastrandrea, LL White (eds) *Climate Change 2014: Impacts, Adaptation, and Vulnerability. Part A: Global and Sectoral Aspects.*

Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom: 411 – 484.

Pörtner H-O, Langenbuch M, Reipschläger A (2004). Biological impact of elevated ocean CO_2 concentrations: lessons from animal physiology and earth history. *Journal of Oceanography* **60**: 705 – 718.

Pörtner H-O, Peck L, Somero G (2007). Thermal limits and adaptation in marine Antarctic ectotherms: an integrative view. *Philosophical Transactions of the Royal Society B: Biological Sciences* **362** (1488): 2233 – 2258.

Price HJ, Boyd KR, Boyd CM (1988). Omnivorous feeding behavior of the Antarctic krill *Euphausia superba*. *Marine Biology* **97**: 67 – 77.

Pyper W (2002). Age riddle confounds fair fishing for krill. *ECOS* **111**: 3.

Quetin LB & Ross, RM (1984). Depth distribution of developing *Euphausia superba* embryos, predicted from sinking rates. *Marine Biology* **79**: 47 – 53.

Ratnarajah L, Nicol S, Bowie AR (2018). Pelagic iron recycling in the Southern Ocean: Exploring the contribution of marine animals. *Frontiers in Marine Science* **5** (109): 1 – 9.

Raven J, Caldeira K, Elderfield H, Hoegh-Guldberg O, Liss P, Riebesell U, Shepherd J, Turley C, Watson A, Heap R, Banes R, Quinn R (2005). *Ocean acidification due to increasing atmospheric carbon dioxide*. The Royal Society, London, United Kingdom.

Riebesell U & Gattuso J-P (2014). Lessons learned from ocean acidification research. *Nature Climate Change* **5** (1): 12 – 14.

Reid K & Arnould JPY (1996). The diet of Antarctic fur seals *Arctocephalus gazella* during the breeding season at South Georgia. *Polar Biology* **16**: 105 – 114.

Reiss CS (2016). Age, growth, mortality, and recruitment of Antarctic krill, *Euphausia superba*. In: V Siegel (ed) *Biology and Ecology of Antarctic Krill (Advances in Polar Ecology)*. Springer International Publishing, Switzerland: 101 – 144.

Reiss CS, Walsh J, Goebel ME (2015). Winter preconditioning determines feeding ecology of *Euphausia superba* in the Antarctic Peninsula. *Marine Ecology Progress Series* **519**: 89 – 101.

Riebesell U, Fabry VJ, Hansson L, Gatusso J-P (2011). Guide to best practices for ocean acidification research and data reporting. Publications Office of the European Union, Luxembourg. doi: 10.2777/66906.

Rintoul SR, Chown SL, DeConto RM, England MH, Fricker HA, Masson-Delmotte V, Naish TR, Siegert MJ, Xavier JC (2018). Choosing the future of Antarctica. *Nature* **558** (7709): 233 – 241.

Rivest EB & Hofmann GE (2015). Effects of temperature and pCO_2 on lipid use and biological parameters of planulae of *Pocillopora damicornis*. *Journal of Experimental Marine Biology and Ecology* **473**: 43 – 52.

Ross PM, Parker L, Byrne M (2016). Transgenerational responses of molluscs and echinoderms to changing ocean conditions. *ICES Journal of Marine Science* **73**: 537 – 549.

Rossoll D, Bermúdez R, Hauss H, Schulz KG, Riebesell U, Sommer U, Winder M (2012). Ocean acidification-induced food quality deterioration constrains trophic transfer. *PLOS ONE* **7** (4): e34737. doi: 10.1371/journal.pone.0034737.

Rowley AF, Vogan CL, Taylor GW, Clare AS (2005). Prostaglandins in non-insectan invertebrates: recent insights and unsolved problems. *The Journal of Experimental Biology* **208**: 3 – 14.

Saba GK, Fraser WR, Saba VS, Iannuzzi RA, Coleman KE, Doney SC, Ducklow HW, Martinson DG, Miles TN, Patterson-Fraser DL, Stammerjohn SE, Steinberg DK, Schofield OM (2014). Winter and spring controls on the summer food web of the coastal West Antarctic Peninsula. *Nature Communications* **5**: 4328. doi: 10.1038/ ncomms5318.

Saba GK, Schofield O, Torres JJ, Ombres EH & Steinberg DK (2012). Increased feeding and nutrient excretion of adult Antarctic krill, *Euphausia superba*, exposed to enhanced carbon dioxide (CO_2). *PLOS ONE* **7** (12): e52224. doi: 10.1371/journal.pone.0052224.

Sales G, Deagle BE, Calura E, Martini P, Biscontin A, De Pittà C, Kawaguchi S, Romualdi C, Meyer B, Costa R, Jarman S (2017). KrillDB: A de novo transcriptome database for the Antarctic krill (*Euphausia superba*). *PLOS ONE* **12** (2): e0171908. doi: 10.1371/journal.pone.0171908.

Schaafsma FL, Kohlbach D, David C, Lange BA, Graeve M, Flores H, van Franeker JA (2017). Spatio-temporal variability in the winter diet of larval and juvenile Antarctic krill, *Euphausia superba*, in ice-covered waters. *Marine Ecology Progress Series* **580**: 101 – 115.

Schmidt K & Atkinson A (2016). Feeding and food processing in Antarctic Krill (*Euphausia superba* Dana). In: V Siegel (ed) *Biology and Ecology of Antarctic Krill (Advances in Polar Ecology)*. Springer International Publishing, Switzerland: 175 – 224.

Schmidt K, Atkinson A, Petzke K, Voss M, Pond DW (2006). Protozoans as a food source for Antarctic krill, *Euphausia superba*: Complementary insights from stomach content, fatty acids, and stable isotopes. *Limnology and Oceanography* **51** (5): 2409 – 2427.

Schmidt K, Atkinson A, Pond DW, Ireland LC (2014). Feeding and overwintering of Antarctic krill across its major habitats: The role of sea ice cover, water depth, and phytoplankton abundance. *Limnology and Oceanography* **59**: 17 – 36.

Schmidtko S, Heywood KJ, Thompson AF, Aoki S (2014). Multidecadal warming of Antarctic waters. *Science* **346** (6214): 1227 – 1231.

Schram JB, McClintock JB, Angus RA, Lawrence JM (2011). Regenerative capacity and biochemical composition of the sea star *Luidia clathrata* (Say) (Echinodermata: Asteroidea) under conditions of near-future ocean acidification. *Journal of Experimental Marine Biology and Ecology* **407** (2): 266 – 274.

Siegel V (2016a). *Biology and Ecology of Antarctic Krill (Advances in Polar Ecology)*. Springer International Publishing, Switzerland.

Siegel V (2016b). Introducing Antarctic Krill *Euphausia superba* Dana, 1850. In: V Siegel (ed) *Biology and Ecology of Antarctic Krill (Advances in Polar Ecology)*. Springer International Publishing, Switzerland: 1 – 19.

Siegel V & Watkins JL (2016). Distribution, Biomass and Demography of Antarctic Krill, *Euphausia superba*. In: V Siegel (ed) *Biology and Ecology of Antarctic Krill (Advances*

in Polar Ecology). Springer International Publishing, Switzerland: 21 – 100.

Sommer U, Paul C, Moustaka-Gouni M (2015). Warming and ocean acidification effects on phytoplankton--from species shifts to size shifts within species in a mesocosm experiment. *PLOS ONE* **10** (5): e0125239. doi: 10.1371/journal.pone.0125239.

Sperfeld E, Mangor-Jensen A, Dalpadado P (2014). Effect of increasing sea water pCO_2 on the northern Atlantic krill species *Nyctiphanes couchii. Marine Biology* **161**: 2359 – 2370.

Sswat M, Stiasny MH, Taucher J, Algueró-Muñiz M, Bach LT, Jutfelt F, Riebesell U, Clemmesen C (2018). Food web changes under ocean acidification promote herring larvae survival. *Nature Ecology and Evolution* **2**: 836 – 840.

Stammerjohn SE, Martinson DG, Smith RC, Iannuzzi RA (2008). Sea ice in the western Antarctic Peninsula region: Spatio-temporal variability from ecological and climate change perspectives. *Deep Sea Research Part II: Topical Studies in Oceanography*, **55** (18): 2041 – 2058.

Stanley-Samuelson DW (1987). Physiological roles of prostaglandins and other eicosanoids in invertebrates. *Biological Bulletin* **173**: 92 – 109.

Steinacher M, Joos F, Frölicher TL, Plattner G-K, Doney SC (2009). Imminent ocean acidification in the Arctic projected with the NCAR global coupled carbon cycle-climate model. *Biogeosciences* **6**: 515 – 533.

Strahl J, Francis DS, Doyle J, Humphrey C, & Fabricius KE (2016). Biochemical responses to ocean acidification contrast between tropical corals with high and low abundances at volcanic carbon dioxide seeps. *ICES Journal of Marine Science* **73** (3): 897 – 909.

Stübing D & Hagen W (2003). Fatty acid biomarker ratios – suitable trophic indicators in Antarctic euphausiids? *Polar Biology* **26**: 774 – 782.

Stübing D, Hagen W, Schmidt K (2003). On the use of lipid biomarkers in marine food web analyses: An experimental case study on the Antarctic krill, *Euphausia superba*. *Limnology and Oceanography* **48** (4): 1685 – 1700.

Suckling CC, Clark MS, Richard J, Morley SA, Thorne MA, Harper EM, Peck LS (2015). Adult acclimation to combined temperature and pH stressors significantly enhances reproductive outcomes compared to short-term exposures. *Journal of Animal Ecology*, **84** (3): 773 – 784.

Sunday JM, Crim RN, Harley CDG, Hart MW (2011). Quantifying rates of evolutionary adaptation in response to ocean acidification. *PLOS ONE* **6** (8): e22881. doi: 10.1371/journal.pone.0022881

Tarling GA & Fielding S (2016). Swarming and behaviour in Antarctic Krill. In: V Siegel (ed) *Biology and Ecology of Antarctic Krill (Advances in Polar Ecology)*. Springer International Publishing, Switzerland: 279 – 320.

Teschke M, Kawaguchi S, Meyer B (2007). Simulated light regimes affect feeding and metabolism of Antarctic krill, *Euphausia superba. Limnology and Oceanography* **52**: 1046 – 1054.

Thomas PG & Ikeda T (1987). Sexual regression, shrinkage, re-maturation and growth of spent female *Euphausia superba* in the laboratory. *Marine Biology* **95**: 357 – 363.

Trathan PN & Hill SL (2016). The importance of krill predation in the Southern Ocean. In: V

Siegel (ed) *Biology and Ecology of Antarctic Krill (Advances in Polar Ecology)*. Springer International Publishing, Switzerland: 321 – 350.

Trathan PN & Reid K (2009). Exploitation of the marine ecosystem in the sub-Antarctic: Historical impacts and current consequences. *Papers and Proceedings of the Royal Society of Tasmania* **143** (1): 9 – 14.

Trivelpiece WZ, Hinke JT, Miller AK, Reiss CS, Trivelpiece SG, Watters GM (2011). Variability in krill biomass links harvesting and climate warming to penguin population changes in Antarctica. *Proceedings of the National Academy of Sciences* **108** (18): 7625 – 7628.

Valles-Regino R, Tate R, Kelaher B, Savins D, Dowell A, Benkendorff K (2015). Ocean warming and CO_2-induced acidification impact the lipid content of a marine predatory gastropod. *Marine Drugs* **13**: 6019 – 6037.

Van Anholt RD (2004). *Dietary fatty acids and the stress response of fish: arachidonic acid in seabream and tilapia*. PhD Thesis. Radboud University of Nijmegen, The Netherlands.

Vaughan DG, Marshall GJ, Connolley WM, Parkinson C, Mulvaney R, Hodgson DA, King JC, Pudsey CJ, Turner J (2003). Recent rapid climate warming on the Antarctic Peninsula. *Climatic Change* **60** (3): 243 – 274.

Verkaik K, Hamel J-F, Mercier A (2016). Carry-over effects of ocean acidification in a cold-water lecithotrophic holothuroid. *Marine Ecology Progress Series* **557**: 189 – 206.

Virtue P, Mayzaud P, Albessard E, Nichols PD (2000) Use of fatty acids as dietary indicators in northern krill, *Meganyctiphanes norvegica*, from northeastern Atlantic, Kattegat, and Mediterranean waters. *Canadian Journal of Fisheries and Aquatic Sciences* **57**: 104 – 114

Virtue P, Meyer B, Freier U, Nichols PD, Jia Z, King R, Virtue J, Swadling KM, Meiners KM, Kawaguchi S (2016). Condition of larval (furcilia VI) and one year old juvenile *Euphausia superba* during the winter–spring transition in East Antarctica. *Deep Sea Research Part II: Topical Studies in Oceanography* **131**: 182 – 188.

Virtue P, Nichols PD, Nicol S, Hosie G (1996). Reproductive trade-off in male Antarctic krill, *Euphausia superba*. *Marine Biology* **126**: 521 – 527.

Virtue P, Nichols PD, Nicol S (1997). Dietary-related mechanisms of survival in *Euphausia superba*: biochemical changes during long-term starvation and bacteria as a possible source of nutrition. In: B Battaglia, J Valencia, DWH Walton (eds) *Antarctic Communities: Species, Structure and Survival*. Cambridge University Press, Cambridge, United Kingdom: 193 – 201.

Virtue P, Nichols PD, Nicol S, Hosie G (1996). Reproductive trade-off in male Antarctic krill, *Euphausia superba. Marine Biology* **126**: 521 – 527.

Virtue P, Nichols PD, Nicol S, McMinn A, Sikes EL (1993a). The lipid composition of *Euphausia superba* Dana in relation to the nutritional value of *Phaeocystis pouchetii* (Hariot) Lagerheim. *Antarctic Science* **5** (2): 169 – 177.

Virtue P, Nicol S, Nichols, PD (1993b). Changes in the digestive gland of *Euphausia superba* during short-term starvation – lipid class, fatty acid and sterol content and composition. *Marine Biology* **117** (3): 441 – 448.

Wang T, Tong S, Liu N, Li F, Wells ML, Gao K (2017). The fatty acid content of plankton is changing in subtropical coastal waters as a result of OA: Results from a mesocosm study.

Marine Environmental Research **132**: 51 – 62.

Ward DFL, Wotherspoon S, Melbourne-Thomas J, Haapkylä J, Johnson CR (2018). Detecting ecological regime shifts from transect data. *Ecological Monographs* **88** (4): 694 – 715.

Watkins JL, Reid K, Ramm D, Zhao XY, Cox M, Skaret G, Fielding S, Wang XL, Niklitschek E (2016). The use of fishing vessels to provide acoustic data on the distribution and abundance of Antarctic krill and other pelagic species. *Fisheries Research* **178**: 93 – 100.

Watkins JL, Buchholz F, Priddle J, Morris DJ, Ricketts C (1992). Variation in reproductive status of Antarctic krill swarms; evidence for a size-related sorting mechanism? *Marine Ecology Progress Series* **82**: 163 – 174.

Weinstein BG, Double M, Gales N, Johnston DW, Friedlaender AS (2017). Identifying overlap between humpback whale foraging grounds and the Antarctic krill fishery. *Biological Conservation 210*: 184 – 191.

White B (2009). Dietary fatty acids. *American Family Physician* **80** (4): 345 – 350.

Whiteley NM (2011). Physiological and ecological responses of crustaceans to ocean acidification. *Marine Ecology Progress Series* **430**: 257 – 271.

Wittmann AC & Pörtner H-O (2013). Sensitivities of extant animal taxa to ocean acidification. *Nature Climate Change* **3** (11): 995 – 1001.

Wong JM, Johnson KM, Kelly MW, Hofmann GE (2018). Transcriptomics reveal transgenerational effects in purple sea urchin embryos: Adult acclimation to upwelling conditions alters the response of their progeny to differential pCO_2 levels. *Molecular Ecology* **27** (5): 1120 – 1137.

Wynn-Edwards C, King R, Davidson A, Wright S, Nichols DP, Wotherspoon S, Kawaguchi S, Virtue P (2014). Species-specific variations in the nutritional quality of Southern Ocean phytoplankton in response to elevated pCO_2. *Water* **6** (6): 1840 – 1859.

Yang G, King RA, Kawaguchi S (2018). Behavioural responses of Antarctic krill (*Euphausia superba*) to CO_2-induced ocean acidification: would krill really notice?. *Polar Biol*ogy **41**: 727 – 732.

Yoshida T, Virtue P, Kawaguchi S, Nichols PD (2011). Factors determining the hatching success of Antarctic krill *Euphausia superba* embryo: lipid and fatty acid composition. *Marine Biology* **158** (10): 2313 – 2325.